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Los Angeles

Brain Behavior Interactions in Avian Models for Speech Disorders

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of
Philosophy in Molecular, Cellular & Integrative Physiology

By

Qianqian Chen

2015

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ABSTRACT OF THE DISSERTATION

Brain Behavior Interactions in Avian Models for Speech Disorders

By

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Doctor of Philosophy in Molecular, Cellular & Integrative Physiology

University of California, Los Angeles, 2015

Professor Stephanie Ann White, Chair

Humans and songbirds are among the rare animal groups that exhibit socially learned vocalizations. These vocal-learning capacities share a reliance on audition and cortico-basal ganglia circuitry, as well as neurogenetic mechanisms. Thus, songbirds can serve as relevant models in which to study the mechanisms of human speech disorders. Mutations in the transcription factors Forkhead box proteins 1 and 2 (*FoxP1*, *FoxP2*) are associated with language disorders in humans. Both genes exhibit similar expression patterns in the cortex and basal ganglia of humans and songbirds, among other brain regions. Here, I examined neural expression patterns of *FoxP1* and *P2* mRNA in two adult songbird species. I found that *FoxP1* and *P2* expression is similarly expressed in both species, including strong mRNA signals for both factors in multiple song control nuclei. With both species, when the birds sing, *FoxP2* is behaviorally down-regulated within the basal ganglia song control nucleus, Area X, over a similar time course, and expression negatively correlates with the amount of singing. This study confirms that in multiple songbird species, *FoxP1* expression highlights song control

regions, and regulation of *FoxP2* is associated with motor control of song. Mutations in contactin associated protein-like 2 (*Cntnap2*), a *FoxP2* target gene, are associated with cortical dysplasia-focal epilepsy, autism spectrum disorder, and specific language impairment. We have previously characterized the expression of *Cntnap2* in zebra finch (*Taeniopygia guttata*), a songbird species. Within the robust nucleus of the arcopallium (RA), the primary vocal motor control nucleus in zebra finch brain, *Cntnap2* expression becomes sexually dimorphic over the course of song learning. RA shares striking similarities with the laryngeal motor cortex, a language control region in the human brain, both in terms of gene expression profiles and in making direct neuronal projections onto the motor neurons that control the muscles of phonation. To further test the function of *Cntnap2*, I developed shRNA constructs to specifically knock down zebra finch *Cntnap2*. I stereotaxically injected an adeno-associated virus (AAV) bearing the shRNA constructs into zebra finch RA to attenuate the *Cntnap2* expression during the sensorimotor phase of vocal learning. I found that knocking down *Cntnap2* in RA caused inaccurate imitation and a high percentage of omission of the tutor song but did not interfere with the bird's ability to modify its song over the course of sensorimotor learning. These results suggest that among *Cntnap2*'s many functions within the nervous system, its expression within the cortical vocal control region alone is critical for accurate vocal imitation. In summary, these studies provide ongoing support for using songbirds to investigate the neurogenetic mechanisms of human speech and language.

The dissertation of Qianqian Chen is approved.

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This dissertation is dedicated to my parents for their love and support.

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Publications and Presentations

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Chapter 1: Expression analysis of speech-related genes
***FoxP1* and *FoxP2* and their relation to singing behavior in**
two songbird species

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Abstract

Humans and songbirds are among the rare animal groups that exhibit socially learned vocalizations; speech and song, respectively. These vocal-learning capacities share a reliance on audition and cortico-basal ganglia circuitry, as well as neurogenetic mechanisms. Notably, the transcription factors Forkhead box proteins 1 and 2 (FoxP1, FoxP2) exhibit similar expression patterns in the cortex and basal ganglia of humans and the zebra finch species of songbird, among other brain regions. Mutations in either gene are associated with language disorders in humans. Experimental knock-down of FoxP2 in the basal ganglia song control region Area X during song development leads to imprecise copying of tutor songs. Moreover, FoxP2 levels decrease naturally within Area X when zebra finches sing. Here, we examined neural expression patterns of *FoxP1* and *P2* mRNA in adult Bengalese finches, a songbird species whose songs exhibit greater sequence complexity and increased reliance on audition for maintaining their quality. We found that *FoxP1* and *P2* expression in Bengalese finches is similar to that in zebra finches, including strong mRNA signals for both factors in multiple song control nuclei and enhancement of *FoxP1* in these regions relative to surrounding brain tissue. As with zebra finches, when Bengalese finches sing, *FoxP2* is behaviorally down-regulated within basal ganglia Area X over a similar time course, and expression negatively correlates with the amount of singing. This study confirms that in multiple songbird species, *FoxP1* expression highlights song control regions, and regulation of *FoxP2* is associated with motor control of song.

Introduction

The importance of the FOXP subfamily of transcription factors in the brain was not clear until *FOXP2* was identified as the monogenetic locus of a speech and language abnormality. Half of the members of a British pedigree, known as the KE family, suffer from a rare communication disorder. Affected members share a single mutation in *FOXP2* which causes a severe impairment in the selection and sequencing of fine orofacial movements (Vargha-Khadem et al., 1998). In addition to articulatory problems, affected individuals have profound deficits in production and comprehension of word inflections and syntactical structure (Alcock et al., 2002). The phenotype resulting from its mutation indicates that FOXP2 is linked to neural pathways underlying speech and language.

FOXP1 is the closest forkhead family member to FOXP2 with which it shares high similarity at the amino acid level (68% identity and 80% similarity between the two human sequences). FOXP1 can heterodimerize with FOXP2 and can repress transcription of similar groups of genes (Li et al.2003; Shu, 2001; Wang, 2003). FOXP1 is also associated with speech and language through multiple cases (Carr et al., 2010; Hamdan et al., 2010; Horn et al., 2010). For example, a patient with a genetic deletion restricted to *FOXP1* exhibits difficulties with verbal expression resembling the phenotype of affected KE family members (Pariani et al., 2009). Besides humans (*Homo sapiens*), no taxon of primates is capable of substantially modifying its vocal repertoire in response to experience. Moreover, most laboratory animals, including rodents, do not learn a substantial portion of their vocalizations (Kikusui et al., 2011; Arriaga et al., 2012; Marht et al.,2013). In striking contrast, thousands of songbird species share the trait of vocal learning with humans, enabling comparison of brain-behavior relationships among them. Zebra finches (*Taeniopygia guttata*) are a well-studied songbird species in which song learning is

sexually dimorphic: juvenile males learn their courtship songs from adult male conspecifics (tutors) whereas females do not produce learned songs. Zebra finch song is composed of notes, syllables, motifs, and bouts. Notes are the smallest unit of song and are defined as a region of a syllable that maintains a temporally continuous frequency pattern. Syllables are composed of one or more notes bounded by a brief period of silence. Motifs are repeated sequences of syllables lasting ~1 second with multiple motifs in succession organized in a bout. Bouts are composed of several motifs bounded by a longer period of silence (Brenowitz et al., 1997; Price, 1979).

Male, but not female, zebra finches possess the full and interconnected suite of cortico-basal ganglia nuclei that underlies song learning and production. Song control circuitry includes the anterior forebrain pathway (AFP) which is important for song learning in juveniles and song maintenance and plasticity in adults, and the posterior descending pathway which is required for song production (Scharff and Nottebohm, 1991; Brainard and Doupe, 2000; Kao et al., 2005). Neurons in HVC (acronym used as a proper name), a premotor vocal control nucleus, directly project to the robust nucleus of the arcopallium (RA) (Nottebohm et al., 1976) and indirectly project to RA through basal ganglia nucleus Area X, medial nucleus of the dorsolateral thalamus (DLM), and the lateral magnocellular nucleus of anterior nidopallium (LMAN) in the AFP. The AFP is homologous to basal ganglia-thalamo-cortical circuit loops in mammals. Area X shares many features characteristic of the mammalian striatum and pallidum, including cell types and connectivity (Gale and Perkel, 2010).

Songbirds and humans also share neurogenetic mechanisms that underlie their vocal learning capacities. *FoxP1* and *FoxP2* exhibit similar expression patterns in the cortex and basal ganglia of humans and zebra finches (Teramitsu et al., 2004). Knock down of FoxP2 in Area X of juvenile zebra finches leads to imprecise copying of the tutor song, suggesting that FoxP2 is

involved in the normal process of vocal learning (Haesler et al., 2007). Moreover, Area X *FoxP2* is behaviorally and socially regulated. Non-singing zebra finches have high levels which decline acutely when males practice their songs alone (termed undirected singing) in the morning, but not when they sing to females (directed singing) (Teramitsu and White, 2006; Hilliard et al., 2012). The down-regulation of *FoxP2* during undirected singing is particularly robust in juvenile zebra finches undergoing sensorimotor learning: the more they practice, the lower their Area X *FoxP2* levels. Interestingly, hearing is required to maintain this negative correlation (Teramitsu et al., 2010). Moreover, coincident with decreased *FoxP2*, vocal variability increases after two hours of undirected singing in both juvenile and adult zebra finches (Hilliard et al., 2012; Miller et al., 2010). These observations have led us to hypothesize that singing-driven decreases in Area X *FoxP2* levels promote vocal variability and motor exploration whereas high levels promote song stabilization (Miller et al., 2010).

Here, we further test the relationship between learned vocal behaviors and *FoxP1* and *FoxP2* gene expression by examining another songbird species, the Bengalese finch (*Lonchura striata domestica*) in which song learning and song control circuitry are also sexually dimorphic, but whose song exhibits features that are distinct from zebra finch song. Adult male zebra finches sing a linear song sequence and thus exhibit a very simple birdsong ‘syntax’, whereas male Bengalese finches generate songs with greater syntactical complexity, (Okanoya, 2004). After deafening, the songs of Bengalese finches degrade faster than those of zebra finches (Okanoya and Yamaguchi, 1997; Woolley and Rubel, 1997), indicating a greater reliance on audition for their song maintenance. These observations suggest that singing-driven decreases in Area X *FoxP2* levels might be more robust in Bengalese finches than in zebra finches. As a

consequence, increases in song variability following song practice might be evident in adult male Bengalese finches.

We therefore tested the following hypotheses: 1) Bengalese finches and zebra finches share similar *FoxP1* and *P2* gene expression patterns; 2) *FoxP2* mRNA is behaviorally regulated in male Bengalese finches; 3) Down-regulation of *FoxP2* within Area X is correlated with the amount of undirected singing in both species; 4) The singing driven-regulation of *FoxP2* within Area X of Bengalese finches is more profound than in zebra finches. 5) Vocal practice promotes song variability in adult male Bengalese finches.

Materials and methods

We conducted *in situ* hybridization on brain tissue from Bengalese finches and zebra finches of both sexes in different behavioral conditions to investigate *FoxP* gene expression patterns, the time course of down-regulation of *FoxP2*, and the relationship between amount of singing and *FoxP2* levels within Area X. A separate group of adult male Bengalese finches was used to investigate song variability following two different behavioral conditions known to alter Area X *FoxP2* levels (Figure 1-1).

Animals and tissues

All animal use was in accordance with NIH guidelines for experiments involving vertebrate animals and approved by the University of California, Los Angeles Institutional Animal Care and Use Committee. Adult male and female zebra finches and Bengalese finches (age > 120d) were taken from our breeding colony (13:11 hour light/dark cycle). After behavioral monitoring (see below), birds were decapitated for collection of brains, which were rapidly extracted and frozen on aluminum floats on liquid nitrogen, then stored at -80°C until use.

Riboprobe preparation and in situ hybridization analysis

FoxP genes are highly conserved among such disparate avian species as zebra finches and chickens (*FoxP1*: zebra finch vs. chicken, identities = 95%; *FoxP2*: zebra finch vs. chicken, identities = 97%). Although the genome of Bengalese finches is not yet available, the similarity of their *FoxP* genes to zebra finch sequences is expected to be even higher based on their closer

phylogenetic relationship. We therefore used riboprobes directed against zebra finch *FoxP1* and *P2* (Teramitsu et al., 2004) to detect these transcripts in both species. The *FoxP1* probe was designed to hybridize to the coding region upstream of the zinc finger domain of zebra finch *FoxP1*: corresponding to 661–998 bp of human *FOXPI* relative to the start codon. The *FoxP2* probe was designed to hybridize to 1870–2127 bp of the zebra finch *FoxP2* relative to the start codon. pCR4-TOPO vector (Invitrogen) with zebra finch *FoxP* cDNA fragments were used for in vitro transcription to generate sense and antisense RNA probes labeled with [³³P]UTP (Perkin-Elmer) using Riboprobe Combination System-T3/T7 (Promega).

Frozen brains were cryosectioned in either the sagittal or coronal plane at 20 µm and adjacent sections were mounted onto 25x75 mm slides (Superfrost, Fisher) in a manner that created seven replicate sets. One set was stained with thionin to enable identification of neuroanatomical structures. The adjacent four sets were exposed to either the *FoxP1* sense, *FoxP1* antisense, *FoxP2* sense or *FoxP2* antisense probes. In situ hybridizations were performed and signals from different brain regions were quantified as previously described (Teramitsu et al., 2004; Teramitsu and White, 2006; Teramitsu et al., 2010). Sections of Bengalese finches were run aligned with sections of zebra finches from the same behavioral conditions to enable direct comparisons. Preliminary analysis of Bengalese finch sections revealed that 1) the distinct expression patterns between brains exposed to either *FoxP1* or *P2* antisense probes were as expected based on prior studies, 2) signals from antisense probes were robust whereas those from sense probes were negligible, and 3) signals were consistent across adjacent brain sections. These results provide confidence that riboprobes designed from zebra finch cDNA also specifically detect *FoxP1* and *P2* in Bengalese finch brain.

Behavioral monitoring and sound recording

Birds were housed individually in sound attenuation chambers (Acoustic Systems) for 2–3 days prior to the behavioral experiments to enable acclimation to the recording environment. Sounds were recorded using Countryman EMW omnidirectional lavalier microphones and digitized using a PreSonus Firepod (44.1 kHz sampling rate, 24 bit depth). Recordings were acquired using Sound Analysis Pro 2011 software (SAP, Tchernichovski et al., 2000).

Behavioral experiments were conducted between 8:00 A.M. and 11:00 A.M., starting at lights on. For *FoxP* gene analysis, birds were sacrificed following the completion of different behavioral paradigms, illustrated in Figure 1-1A and described as follows: Female birds were left alone and undisturbed inside the chamber for 2h after lights on. Non-singing males (referred to NS, Figure 1-1A) were also left alone for 2h after lights on, but with the door to the chamber ajar. If they appeared to attempt to sing, they were distracted by the experimenter. Those that sang more than 5 motifs despite the experimenter's presence were excluded from this group. Of note, we previously found that the non-singing paradigm did not lead to detectable changes in zebra finch stress levels as measured by serum corticosterone values (Miller et al., 2008). In addition, Area X gene expression patterns from birds that were distracted from singing by an experimenter clustered together with patterns from birds that sang very little by their own volition. This suggests that singing behavior –and not the absence or presence of the experimenter– is the more critical determinate of gene expression in Area X (Hilliard et al., 2012). Males singing undirected song (referred to as UD, Figure 1-1A) were allowed to sing alone inside the chamber for a pre-determined period of time, either 1h, 1.5h or 2h after the first song in the morning. For analysis of song variability, a separate set of birds was used for which the behavioral conditions are illustrated in Figure 1-1B. One group of male birds (n = 6) was kept from singing for two hours and then allowed to sing undirected songs. Songs sung during the subsequent 20 minutes

(termed NS-UD songs) were analyzed. On another day, the same group of male birds was allowed to sing undisturbed for two hours then songs that were sung in the subsequent twenty minutes (termed UD-UD) were analyzed.

Quantification of the amount of singing

Audio files generated by SAP were edited with Audacity 1.3 Beta (<http://audacity.sourceforge.net>) by manual removal of cage noise and calls, leaving only songs. In our previous study on zebra finches, the amount of singing was quantified by counting the number of motifs (Teramitsu and White, 2006). However, there is considerable variability in phonology and macroscopic song structure both within and between the two songbird species studied here (Figure 1-2A). The greater syntactical variability in Bengalese finch song makes it challenging to identify their motifs (Figure 1-2B). Moreover, the length of the motifs varies among different Bengalese finches and between the two songbird species. To minimize error and avoid introducing bias by manually identifying song motifs, we used SAP to automatically measure the length of each song syllable. Syllables were segmented using experimenter-derived amplitude thresholds in SAP, and then run through the “Feature Batch” module, which computes the duration of each syllable in the batch. The total amount of singing was then defined as the sum of the durations of all syllables identified for a given time period.

Quantification of FoxP gene expression

For semi-quantitative and quantitative analyses, optical density (OD) measurements of *FoxP* signals were obtained from digitized images of autoradiograms using Adobe Photoshop 7.0. First, to provide a qualitative comparison of gene expression levels across brain regions, OD values from each region were calculated from multiple sagittal sections of the brains of one 2h UD Bengalese male, one 2h NS Bengalese male (shown in Figure 1-3) and one Bengalese female (shown in Figure 1-4). All OD values were normalized to those from a nidopallial (N) area of the same section that did not contain any song control nuclei. Values are reported in Table 1-1.

For quantitative analysis of Area X *FoxP2* levels, OD values from within Area X were normalized to those from the ventral striato-pallidum (VSP), as previously described (Teramitsu and White, 2006). To determine the statistical significance of the Area X *FoxP2* levels, a resampling procedure was employed as follows: 10,000 hypothetical data sets of the same n were resampled from the actual normalized OD values and the amount of singing in the experiments. For each resampled data set, a slope of the linear regression of these variables (OD vs. amount of singing) was calculated, generating a distribution of 10,000 slopes for each species. A correlation was determined to be significantly negative if the upper and lower boundaries of the 95% confidence interval for the distribution of slopes were negative.

Syllable identification and clustering

All syllable clustering and sequence analysis was performed in the freely available R programming language (<http://www.r-project.org>) using custom-written clustering and syntax entropy scripts, available at the White lab website (<https://www.ibp.ucla.edu/research/white/code.html>).

To group syllables in an unbiased fashion and subsequently calculate syntax entropy, a hierarchical clustering and automated tree-trimming algorithm were utilized. Raw acoustic recordings from the first 20 minutes following NS or UD for each bird were subjected to SAP's "Feature Batch" utilizing experimenter-derived amplitude thresholds to segment syllables. A number of filtration steps were then applied to the "Feature Batch" output to identify song syllables from non-song sounds (wing flaps, cage noise, etc.) captured by the recording software. An initial filtration step implemented user defined duration thresholds above and below which all sounds were removed from the data set. Next, a maximum inter-syllable interval (ISI) was determined by the experimenter for all remaining prospective syllables in the dataset. Syllables that fell below this ISI of each other were grouped into prospective motifs/bouts. A filter to remove all motifs/bouts composed of two and/or three syllables was then applied. WAV files representing each motif/bout were generated and presented to the user for visual inspection, at which point motifs consisting of calls or non-song sounds in the recordings were removed from the dataset if present. Finally, individual WAV files for all remaining syllables were generated.

Individual WAV files for both behavioral sessions for each animal were run against themselves in SAP's "Similarity Batch" module in an $M \times N$ symmetric similarity batch. Upon completion of the batch, the product of the similarity and accuracy score for each syllable-syllable comparison was calculated and stored in a square matrix with rows and columns representing individual syllables and the elements of the matrix representing the product of the similarity and accuracy scores for a given syllable-syllable comparison. A distance matrix was then created by calculating the Euclidean distance between the product of similarity and accuracy scores for all syllable-syllable pairs. This distance matrix was used as the input to a hierarchical clustering function in the WGCNA R package (Langfelder and Horvath, 2008),

generating a dendrogram. Branches of the dendrogram were then pruned using the dynamic tree-trimming algorithm, also in the WGCNA R package, a novel method for detecting clusters within hierarchical trees by considering the shape of the branches when trimming them into groups (Langfelder et al., 2008). Upon completion of cluster detection, each cluster is described by an “eigensyllable”, defined as the first principal component of the cluster as determined by singular value decomposition. The Pearson correlation between all module eigensyllables was then computed and clusters whose eigensyllables correlated above a user-defined threshold (in this case, 0.75) were merged, generating the final number of clusters/syllable types in each bird’s song.

Final inspection of cluster homogeneity was performed by visual inspection of syllable spectrograms within each cluster. Syllables inappropriately assigned to a cluster were manually reassigned.

Syntax entropy

The syllable syntax, defined as the sequence in which the bird orders its syllables, was determined based on syllable cluster assignment in the preceding step. Syntax entropy was then calculated as described in Miller et al., (2010). A string-based approach was utilized for syntax analysis, as motifs were often difficult to identify in Bengalese finch songs. Values for syllable syntax entropy reported are weighted entropy scores, which are adjusted for the frequency of occurrence of each syllable type when determining its contribution to overall syntactical entropy. A resampling paired T-test was utilized to assess the significance of change in syntax entropy scores between behavioral conditions for all birds as a group.

Similarity, accuracy, identity, and syllable acoustic features

Upon completion of clustering, syllables within each cluster were divided into NS-UD and UD-UD groups. All syllable types that did not have at least 20 renditions sung in both behavioral contexts were removed from consideration in analysis of acoustic features. The range in the number of renditions for the remaining syllables that were analyzed was 55 – 762. A bootstrap one-way ANOVA was performed on similarity, accuracy, and identity scores and all acoustic features within each bird to determine whether syllables were independent of one another. For all acoustic measures, the between syllable difference p-value was less than 0.05, thus syllables were treated as independent of one another.

Resampling two-way ANOVAs were performed for each acoustic measure using syllables and behavioral condition as the two independent factors. F-statistics were generated for the actual data set and then compared to a distribution of 10,000 F-statistics calculated by resampling the original data under assumption of the null hypothesis to determine if a syllable effect, a behavioral effect, and/or an interaction between the two variables were present for each measure.

Results

FoxP1 expression in Bengalese finch brain

FoxP1 mRNA signals indicated high expression levels in the densocellular part of the hyperpallium (HD), the mesopallium (M), the striato-pallidum and the dorsal thalamus, in both male (Figure 1-3) and female (Figure 1-4) Bengalese finches. In the basorostral pallial nucleus (Bas) and song control nucleus LMAN, *FoxP1* expression was lower than in the surrounding nidopallium (N) region regardless of sex (Figure 1-3C, Figure 1-4). In contrast, sexually dimorphic *FoxP1* expression was observed in song control nuclei HVC, RA and striato-pallidal Area X, as the signals were greater in these nuclei relative to the respective surrounding brain tissue only in male Bengalese finches (Figure 1-3C). In females, signals were similar across these sub-regions (Figure 1-4). *FoxP1* did not appear to be regulated by undirected singing in male Bengalese finches. Expression patterns from sagittal sections containing multiple song control regions were broadly similar between the 2h NS and UD groups (Figure 1-3C). A semi-quantitative summary of these observations is presented in Table 1-1. Coronal sections from a separate set of birds were used to focus on Area X and LMAN but again, no behavioral regulation of *FoxP1* was observed.

FoxP2 expression in Bengalese finch brain

FoxP2 signals were lightly and uniformly distributed in cortical areas whereas they were robust in the striato-pallidum, the dorsal thalamus and the Purkinje cell layer of the cerebellum in

both male (Figure 1-3) and female (Figure 1-4). Bengalese finches. No sexual dimorphism of FoxP2 expression was observed in any of the song control nuclei except for Area X. FoxP2 expression within Area X in female Bengalese finches was similar as that of the surrounding striato-pallidum (Figure 1-4). FoxP2 expression in Area X of male Bengalese finches, has reported to be lower than the surrounding striato-pallidum (Haesler, 2004). However, the behavioral condition of the birds used in that experiment was not specified. Here we present evidence that FoxP2 within Area X is comparable to or slightly higher than surrounding striato-pallidum in 2h NS Bengalese finches but lower in 2h UD Bengalese finches (Figure 1-3D). A semi-quantitative summary of these observations is presented in Table 1-1.

Behavioral regulation of FoxP2 within Bengalese finch Area X

In zebra finches, *FoxP2* expression levels decline specifically within Area X when males engage in 2h of UD singing in the morning (Hilliard et al., 2012; Teramitsu and White, 2006; Teramitsu et al., 2010). To determine whether similar singing-driven changes occur in a related songbird species with distinct song features, we examined *FoxP2* expression in Area X of male Bengalese finches, in parallel with that in zebra finches, and compared levels between UD and NS conditions. To confirm the behavioral regulation of *FoxP2* suggested in Figure 1-2D, additional 2h NS and 2h UD male Bengalese finches were sacrificed and brain tissues were sectioned coronally to display Area X bilaterally in the same section. The additional time points of 1h UD and 1.5h UD groups were utilized to track the time course of down-regulation of *FoxP2* within Area X during singing. We found that Area X *FoxP2* levels were significantly down-regulated at the 1.5h UD and 2h UD time points for both species (Figure 1-6).

FoxP2 levels within Area X in 2h UD Bengalese finches were significantly higher than those found in 2h UD zebra finches ($p < 0.01$). In order to interpret this difference, we measured the amount of singing in both groups. We found that zebra finches in our study sang more than Bengalese finches did (Bengalese finch mean \pm SEM of 351 \pm 53s vs. zebra finch mean \pm SEM of 758 \pm 166s, Kruskal–Wallis nonparametric test, $p = 0.040$). Thus, the difference of *FoxP2* levels between 2h UD Bengalese finches and 2h UD zebra finches could reflect the difference in the amount of singing. To explore this possibility, the relationship between the amount of singing and *FoxP2* levels was further examined.

Correlation between FoxP2 levels and amount of singing

Area X *FoxP2* levels were negatively correlated with the amount of singing in both zebra and Bengalese finches, as illustrated by the negative slope of the linear regression lines that were fit to the data from each species (ZF: $p < 0.0002$; BF: $p < 0.0003$; Figure 1-7). These results indicate that the more a given bird sang, the lower its Area X *FoxP2* level. There was no statistically significant difference between the slopes of the two regression lines ($p > 0.05$, see below), indicating that, contrary to our prediction, Bengalese finch *FoxP2* levels within Area X are not more responsive to singing than those in zebra finches.

Song variability after vocal practice

Songs that were sung by adult male Bengalese finches in the 20-minute period immediately following a 2h period of UD singing (UD-UD) were compared with those sung following 2h of non-singing (NS-UD). One expectation is that there would be no difference between the behavioral conditions, based on prior work in zebra finches in which a difference

was only observed in juveniles. The other expectation is that variability after UD-UD singing would be increased relative to the NS-UD conditions, based on the overall greater variability in Bengalese song and its strong dependence on hearing. In line with a majority of our predictions, we found that for many phonological and sequential measures of song variability there were no differences between the two conditions. However, on certain measures, a slight decrease in variability was observed in the UD-UD condition relative to the NS-UD condition, in contrast to our predictions. To describe syllable variability, we examined the average within group similarity, accuracy, and syllable identity (similarity x accuracy/100) of all syllables within a cluster analyzed as a function of behavioral condition. The variability of syllable identity ($p=0.034$,) was lower in the UD-UD condition, reflecting similar trends in similarity ($p=0.080$) and accuracy ($p=0.075$). We next examined the mean coefficient of variance (CV) for all syllables within a cluster. Again contrary to our predictions, the CV was lower in the UD-UD condition for individual syllable features of pitch goodness ($p=0.0002$), Wiener entropy ($p=0.004$) and mean frequency ($p=0.017$; Table 1-2). A two way ANOVA revealed that there was no effect of behavioral condition on the mean values for any of these features. Finally, we utilized entropy-based methods similar to Miller et al., (2010) to measure syntax variability, investigating all syllables produced during the 20 minutes following each behavioral condition using a string-based analysis described in that study. The results indicate no significant difference in syntax entropy between the two behavioral conditions (average NS-UD entropy=0.185, average UD-UD entropy=0.168; $p>0.05$), similar to our prior findings in adult zebra finches.

Discussion

Sexually dimorphic expression of FoxP1 in songbirds

In line with our expectations, the brain expression patterns of *FoxP1* and *FoxP2* in Bengalese finches are broadly consistent with those previously described in zebra finches (Teramitsu et al., 2004), including strong mRNA signals for both factors in multiple song control nuclei and enhancement of *FoxP1* in HVC and Area X relative to surrounding brain tissue. One apparent difference between the two species was in the arcopallial song control region, RA. *FoxP1* in the RA of female zebra finches is higher relative to the surrounding brain tissue (Teramitsu et al. 2004), but this enhancement was not prominent in coronal sections of a female Bengalese finch brain (data not shown) and was not detected in sagittal sections of another female Bengalese finch (Figure 1-4). Whether or not this is a true species difference is unclear because we were unable to detect RA in the Nissl stained female Bengalese finch sections, despite its visibility in sections from male brains subjected to the same staining conditions (Figure 1-3). As previously reported in zebra finches, the RA of male Bengalese finches exhibited *FoxP1* signals that were slightly higher than those of the surrounding arcopallium. Projection neurons of RA synapse directly onto the motor neurons that innervate the muscles of phonation, similar to direct projections of layer V motor cortical neurons onto laryngeal motor neurons in humans, and thought to enable the capacity for vocal learning (Jürgens, 2009; Arriaga et al., 2012). In spinal cord, FOXP1 plays a critical role in defining the columnar identity of motor neurons at each axial position, as well as organizing motor axon projections (Rousso et al., 2008). Similarly, FoxP1 may organize the RA cortical motor neuron projection to syringeal and respiratory motor neurons in songbirds.

With regard to other telencephalic song control regions, enhanced expression of *FoxP1* in HVC and Area X in male, but not female, Bengalese finches, mirrors the zebra finch expression pattern. There is no evidence for singing-driven regulation for *FoxP1* expression in either adult Bengalese or zebra finch brains (Figure 1-3, Figure 1-5, Table 1-1). The sexually dimorphic expression of *FoxP1* in song control areas (HVC, male RA, Area X), together with the speech and language deficits associated with its mutation in humans (Carr et al., 2010; Hamdan et al., 2010; Horn et al., 2010; Pariani et al., 2009) suggest that FoxP1 plays a role in the formation of song circuitry dedicated to singing behavior.

The expression of *FoxP1* within LMAN and Bas in Bengalese finches is low relative to surrounding tissue, and doesn't exhibit sexually dimorphic patterns nor singing-driven regulation. Bas is involved in feeding and oral-manipulative behaviors other than vocalization and does not anatomically connect to the vocal control system in songbirds (Wild and Farabaugh, 1996). Since both male and female finches engage in oral movements related to feeding behavior, it is not surprising that *FoxP1* levels in Bas are similar in both sexes. In contrast, LMAN plays a key role only in male song learning and maintenance (Bottjer et al. 1984; Brainard and Doupe, 2000), yet *FoxP1* mRNA expression was not sexually dimorphic in this nucleus. Further investigation may determine whether FoxP1 protein exhibits sexual dimorphism in LMAN as differences between transcriptional and translational levels have been observed for other transcription factors in song control circuitry (Whitney and Johnson, 2005). Although FOXP1 mutations in humans are accompanied by language disorders, the impact of FoxP1 on song learning and production remains to be determined. Given that we did not observe behavioral regulation of *FoxP1* in either species, it seems likely that its role may be in promoting the developmental differentiation of

neural structures, consistent with the general role of Fox transcription factors during embryogenesis (Carlsson and Mahlapuu, 2002).

Behavioral regulation of FoxP2 in songbirds

Unlike *FoxP1*, *FoxP2* expression in male songbirds was not enriched in HVC and RA, and appeared similar to levels in HVC and RA of female brains (Figure 1-3, Figure 1-4, Table 1-1). In Area X, *FoxP2* was slightly higher or comparable to levels in the adjacent VSP in NS adult male songbirds. *FoxP2* expression is enhanced in the striato-pallidum of hatchling zebra finches and increases in Area X during development (Teramitsu et al., 2004). This observation, together with the structural deficits in the basal ganglia of affected KE family members, is consistent with a role for FoxP2 in contributing to the structural organization of basal ganglia regions critical for vocal learning. Post-embryogenesis, Area X *FoxP2* levels are down-regulated after undirected singing in juvenile and adult zebra finches (Teramitsu and White, 2006) (Teramitsu et al., 2010). Lentiviral-mediated FoxP2 knockdown in Area X of juvenile zebra finches results in inaccurate copying of the tutor song (Haesler et al., 2007). Together, these findings suggest that FoxP2 is not only involved in forming neural structures for vocal learning during embryogenesis, but also in the ongoing use of such structures during vocal learning and adult song maintenance including in adult male Bengalese finches.

Correlation between Area X FoxP2 levels and undirected singing in two species of songbird

We investigated the time course over which *FoxP2* levels are first observed to decrease in Area X during singing in both Bengalese and zebra finches. We found that levels became significantly down-regulated at the 1.5h time-point in both species (Figure 1-6). Contrary to our prediction, Area X *FoxP2* down-regulation in Bengalese finches was not more robust than in zebra finches. This outcome is qualified by the recognition that experimental quantification of the amount of singing is not always proportional to the time spent singing. For example, one zebra finch sang for 241s within 2h whereas another sang for 487s within 1h. We observed a negative correlation between the amount of singing and *FoxP2* levels within Area X of zebra finches, which confirms our prior studies (Hilliard et al., 2012; Teramitsu and White, 2006; Teramitsu et al., 2010). We now report a similar negative correlation in Bengalese finches (Figure 1-7B). Thus, singing may promote *FoxP2* mRNA degradation, possibly through miRNA regulation (Clovis et al., 2012) or inhibit mRNA synthesis following song onset. In either case, this regulation of *FoxP2* takes time, only producing significant decreases 1.5 hours following song onset in this study (Figure 1-6). It is difficult to disentangle the effects of time and the amount of singing on *FoxP2* levels because we cannot control the amount and timing of singing once birds start. For each species, in birds that did sing similar amounts of song (Figure 1-7), there is a trend that the longer time they were given before being sacrificed, the lower their Area X *FoxP2* levels.

FoxP2 down-regulation within Area X in Bengalese finches and zebra finches

When all birds are considered, the down-regulation of *FoxP2* did not occur on a faster time scale in Bengalese finches than in zebra finches as demonstrated by the lack of a

statistically significant difference in the slopes of regression lines plotted to the data (Figure 1-7). The lack of a detectable difference between the two species may be due to a lack of sensitivity in the in situ hybridization. However, in pilot experiments, we compared *FoxP2* levels obtained with quantitative reverse transcriptase PCR from cDNA obtained from unilateral punches of Area X, with those obtained from in situ hybridization of the remaining hemi-sections from the same bird (J. Liu, unpublished honors thesis). The sensitivity was comparable across methods, indicating the suitability of our approach, which also enables us to compare our current findings with past studies that employed in situ analyses. The relationship between *FoxP2* and singing in Bengalese finches may be underestimated here simply because they sang less as a group. A broader range of singing might enable detection of more subtle differences between the species. Alternatively, the dependence of *FoxP2* levels on singing may indeed be similar in both species, despite differences in features of their songs.

Vocal variability after vocal practice

We previously found that in juvenile (75 d) zebra finches, vocal practice for 2h in the morning leads to increased vocal variability (Miller et al., 2010) and that in adult zebra finches, the amount of singing is correlated with increased spectral entropy (Hilliard et al., 2012). Thus, we predicted that vocal practice might lead to increased vocal variability in adult Bengalese finches. To our surprise, we found that despite similar behavioral regulation of *FoxP2* in Bengalese and zebra finches, periods of low *FoxP2* are associated with slight decreases in variability of multiple features in Bengalese finch song (Miller et al., 2010; Hilliard et al., 2012). Thus, it is possible that *FoxP2* down regulation may decrease vocal variability or that changes in

FoxP2 levels are unrelated to changes in vocal variability in this species. Arguing against these possibilities is the observation that viral knockdown of *FoxP2* in Area X is sufficient to increase variability in both juvenile (Haesler et al, 2007) and adult zebra finches (Murugan and Mooney, 2012). Multiple factors could contribute to the observed difference in these select song features, as follows.

The amount of singing done by each species could influence whether song is more or less variable in the UD-UD condition. Bengalese finches in our study sang roughly half as much as the zebra finches and the corresponding down regulation of *FoxP2* is about half the magnitude. It is possible that *FoxP2* levels must drop below a critical threshold in order to de-repress gene transcription and initiate molecular changes that lead to increased variability, or that the amount of singing by Bengalese finches was sufficient to down regulate *FoxP2* mRNA but not the protein (Miller et al., 2008). These possibilities could be supported by examining Bengalese song after more extended bouts (~4 hr) of UD singing, however, this may be confounded by the fact that *FoxP2* levels vary as a function of both the amount of singing and the total time allotted for singing (Figure 1-7).

The age of the Bengalese finches used here (>300 d) may present another confound in our ability to detect differences in vocal variability between NS-UD vs. UD-UD birds. Increased song variability was previously observed in younger adult zebra finches (n=18 between 120d-200d; Hilliard et al. 2012) correlated to the amount of song. Both Bengalese and zebra finches undergo age related changes in vocal quality and the ability to exhibit vocal plasticity (Brainard and Doupe, 2001; Cooper et al., 2012), thus they may undergo age related changes in how molecular microcircuits impact behavior. Further, age and species related differences in basal vocal variability may have statistically limited our ability to detect these changes. In zebra

finches, our ability to detect acute regulation of vocal variability was limited to 75d juvenile birds as 65d and a group of 6 adult birds showed too much and too little variability, respectively, to derive adequate statistical power (Miller et al., 2010). A follow up study found that statistical power was achieved when the number of adult zebra finches was increased to 18 UD singers with higher numbers of motifs uttered in the 2h being correlated with increased song variability (Figure 1-3B, Hilliard et al. 2012).

In summary, these data indicate that *FoxP1* is enriched in most song control nuclei of male Bengalese finches, with the notable exception of LMAN, similar to its expression pattern in zebra finches. No singing driven regulation of this transcription factor was observed in either species, suggesting a sexually dimorphic role in formation of brain structures that support vocal learning in songbirds. In contrast, *FoxP2* levels in Area X do exhibit singing-driven decreases in both species, with a similar dependence on both the amount of singing and the time since song onset, with the caveat that Bengalese finches in our study sang less than zebra finches. The impact of this down-regulation in zebra finches appears to be to increase vocal motor exploration, particularly during song learning and as evidenced by multiple prior studies. Here, in Bengalese finches, we did not observe a similar relationship, which could reflect a true species difference. We deem it more likely that the differences in age and amount of singing of the Bengalese finches in our study relative to the zebra finches precluded detection of this relationship. Future work in songbirds to examine protein expression of these factors as well as to genetically intervene in their expression promise to illuminate organizational versus activational functions of these molecules related to human language.

List of abbreviations

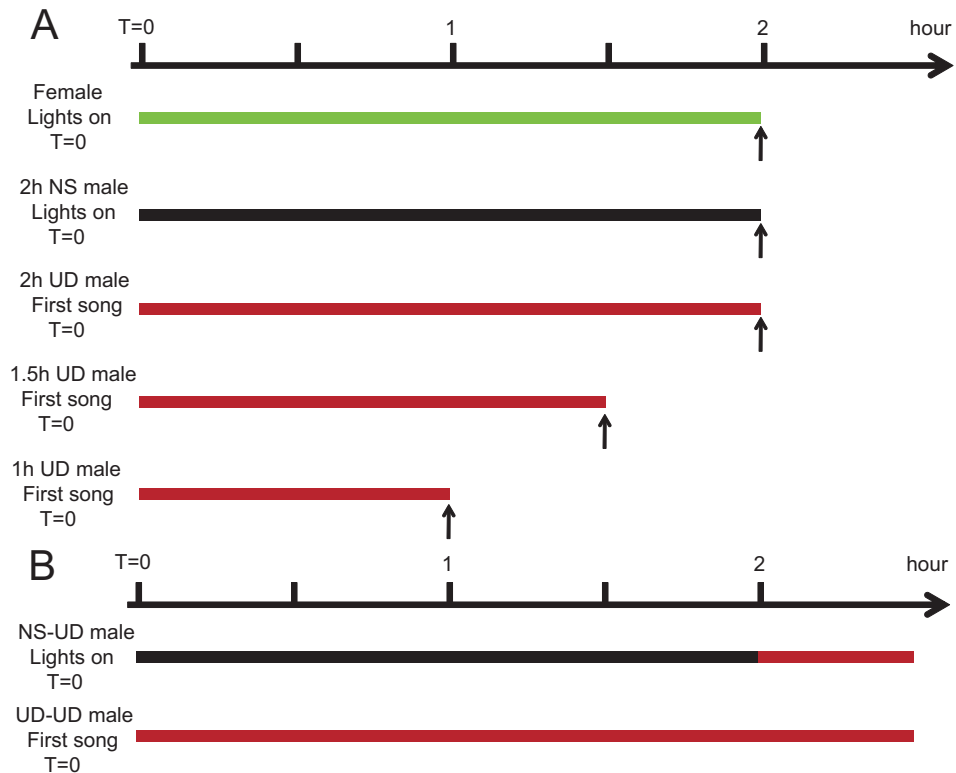
A	Arcopallium
AFP	Anterior forebrain pathway
B, Bas	Basorostral pallial nucleus
BF	Bengalese finch
DLM	Medial nucleus of the dorsolateral thalamus
HA	Apical part of the hyperpallium
HD	Densocellular part of the hyperpallium
HVC	letter-based name, located in nidopallium
LMAN	Lateral magnocellular nucleus of anterior nidopallium
M	Mesopallium
N	Nidopallium
NC	Caudal nidopallium
NS	Non-singing
RA	Robust nucleus of arcopallium
StL	Lateral striatum
StM	Medial striatum
UD	Undirected singing
VSP	Ventral striato-pallidum
X	Area X
ZF	Zebra finch

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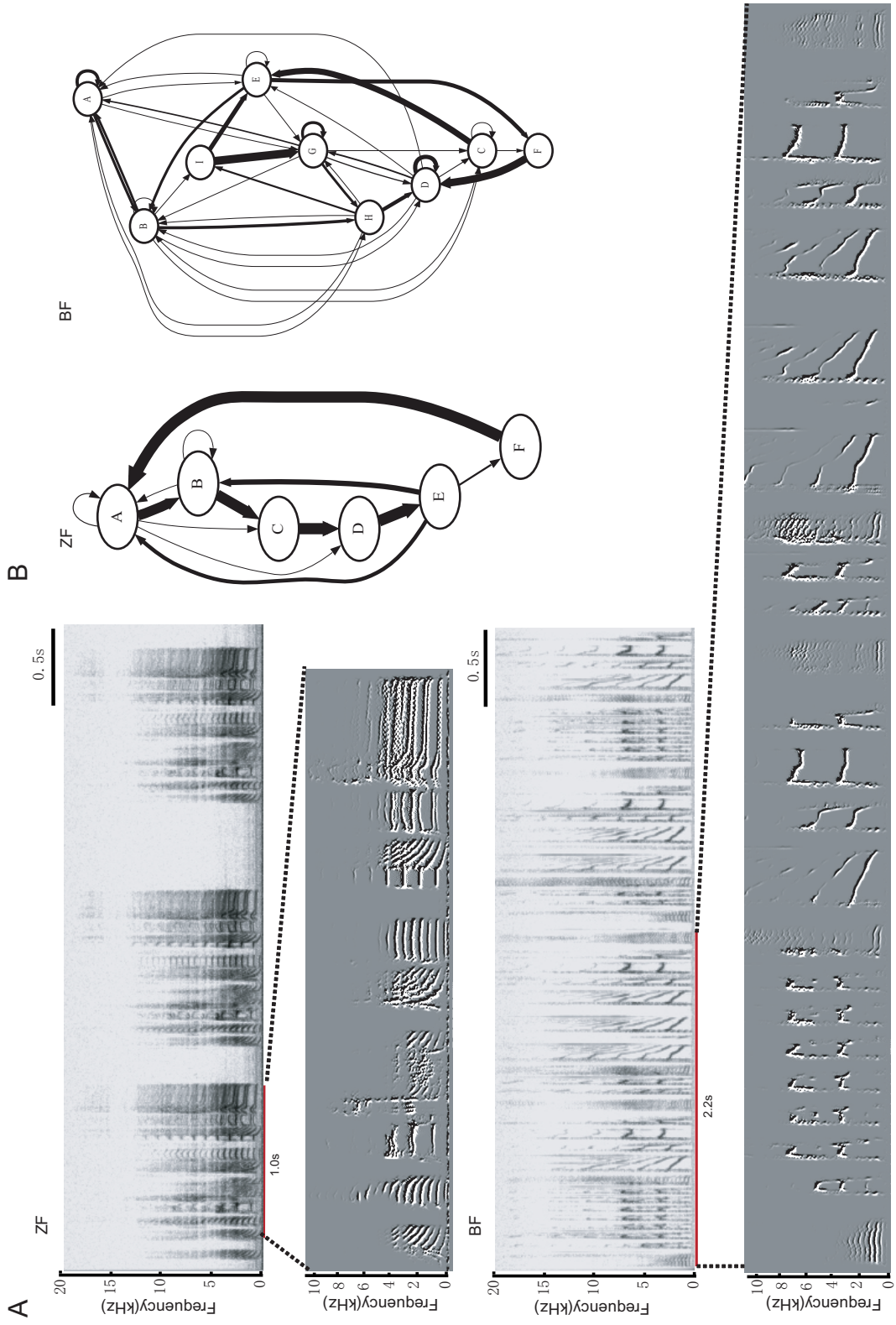
Figures

Figure 1-1: Timelines for the behavioral groups used in this study.



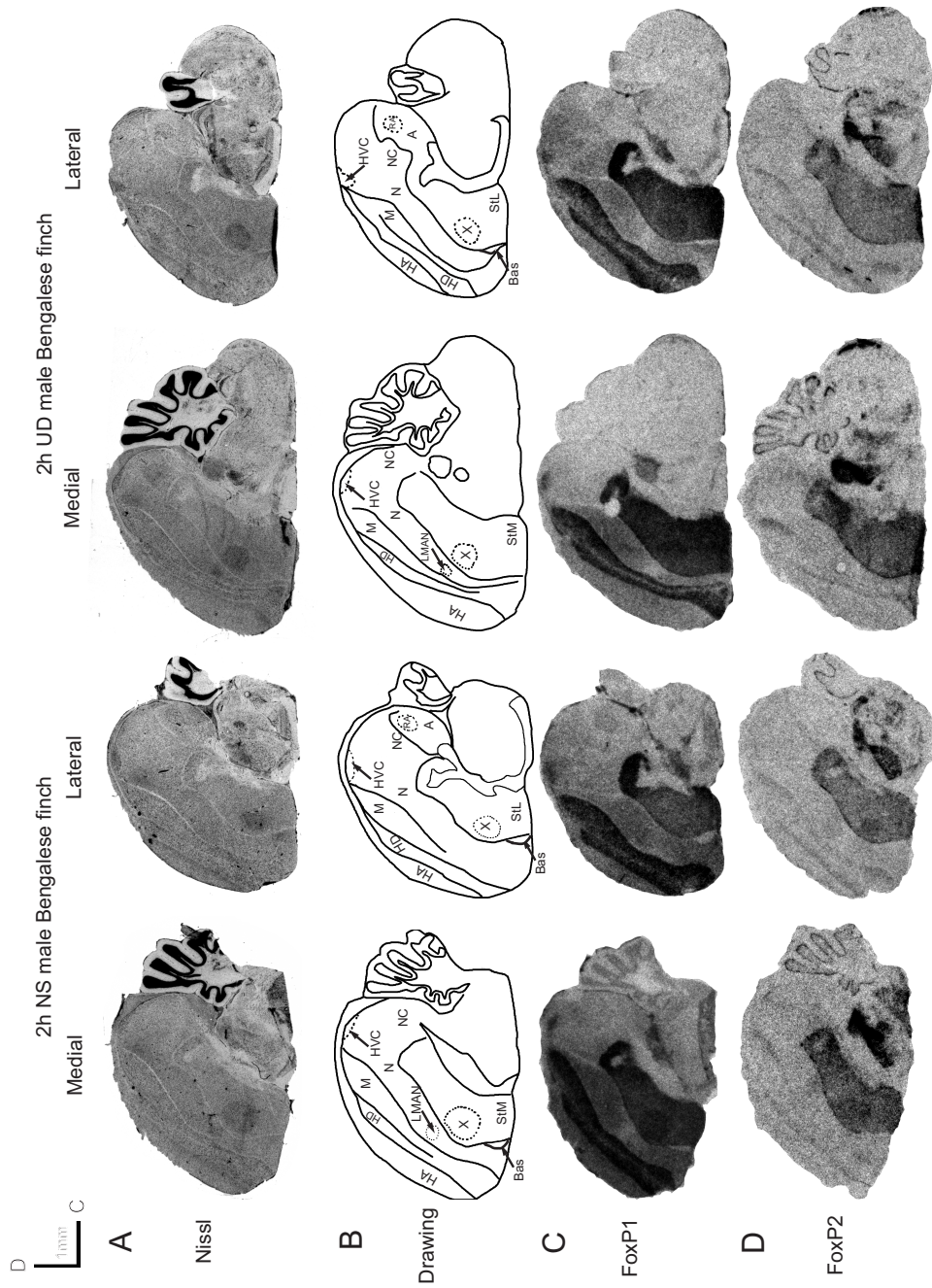
A) Experimental design for time-course analysis of *FoxP1* and *FoxP2* behavioral regulation. On the day of the experiment, female birds remained alone in sound attenuation chambers for 2h (green bar). NS males were discouraged from singing by the experimenter sitting nearby for 2h (black bar). UD males sang alone in the isolation chamber for variable periods of time (red bars). Arrows indicate the timepoints of sacrifice. B) Experimental design for song variability analysis. Songs sung after the 2h timepoint were analyzed for song variability. Birds were not sacrificed in this experiment.

Figure 1-2: Representative exemplars of zebra and Bengalese finch song.



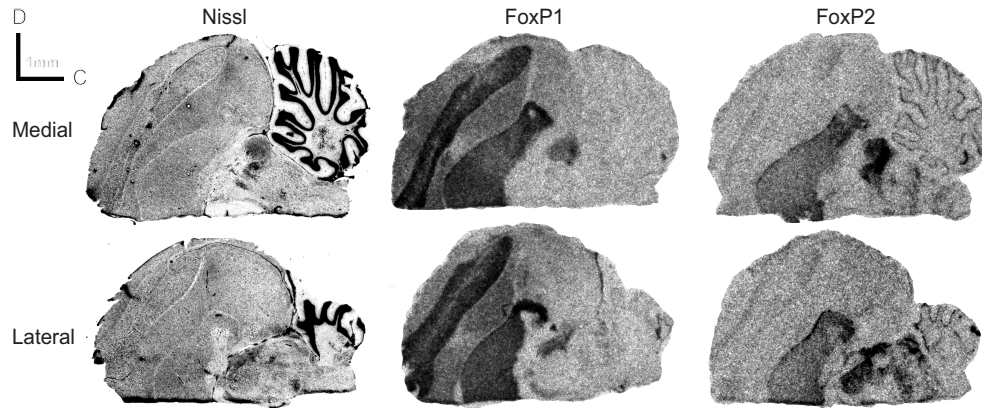
A) Spectrograms from a male zebra finch (ZF, top) and a male Bengalese finch (BF, middle) are shown. The red bar underneath each spectrogram indicates the length of one motif. Spectral derivatives of these motifs are shown underneath each spectrogram. B) Markov chains generated from zebra finch and Bengalese finch songs. Letters denote syllables. Lines represent the probability of syllable transitions. Thicker lines indicate greater probabilities.

Figure 1-3: Representative bright-field photomicrographs of *FoxP1* and *FoxP2* mRNA expression patterns in a series of sagittal sections from one 2h NS (left) and one 2h UD (right) adult male Bengalese finch brain.



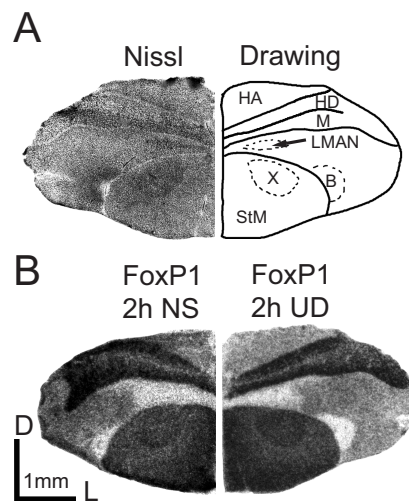
Both medial and lateral sections are shown to enable display of the song control nuclei investigated here. A) Nissl-stained sagittal sections. Locations of medial and lateral sections correspond to the level of sagittal plates 6 and 11 in the zebra finch atlas of Nixdorf-Bergweiler and Bischof (2007), respectively. B) Schematic drawings based on the Nissl stains. C) *FoxP1* mRNA signals. D) *FoxP2* mRNA signals. Medial sections in A, C and D were adjacent or near adjacent to one another; similarly, lateral sections were adjacent or near adjacent. Scale bar 1mm. D, dorsal; C, caudal.

Figure 1-4: Representative bright-field photomicrographs of *FoxP1* and *FoxP2* mRNA expression patterns in a pair of sagittal sections from adult female Bengalese finch brain.



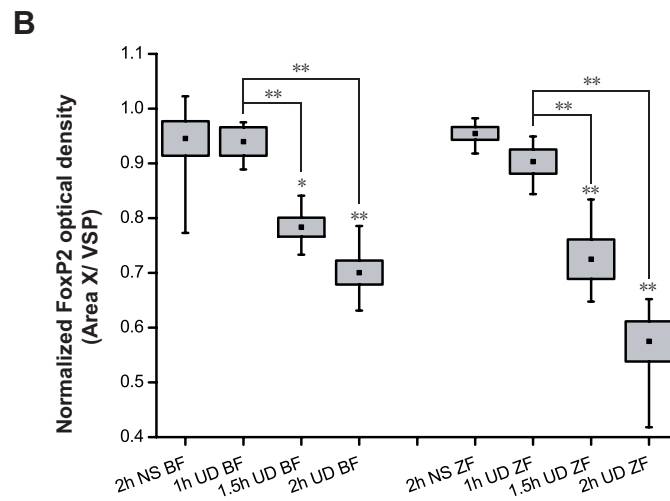
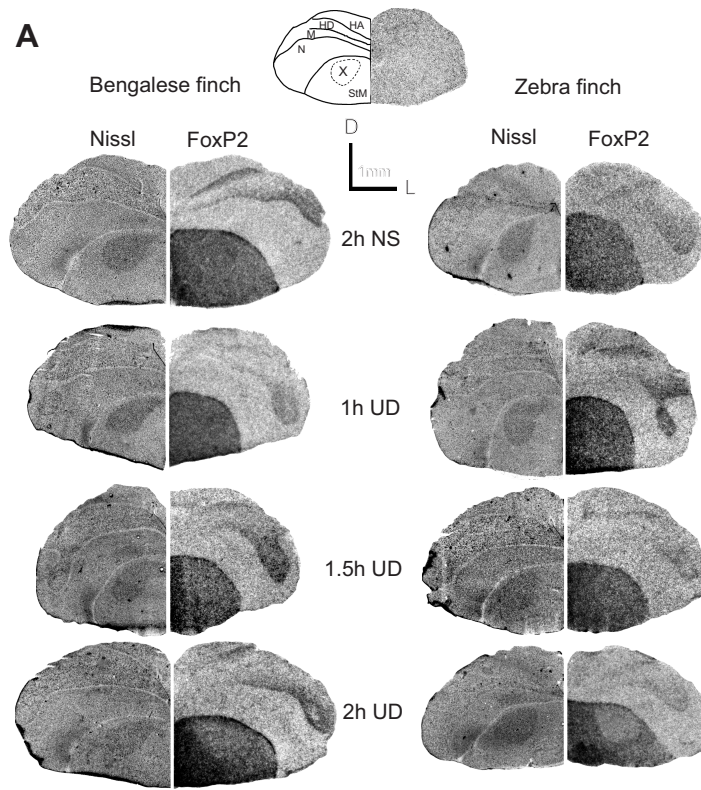
Locations of medial and lateral sections correspond to the level of sagittal plates 6 and 11 in the zebra finch atlas of Nixdorf-Bergweiler and Bischof (2007), respectively. Medial plate shows HVC and LMAN and lateral plate shows HVC and RA in corresponding sections from male birds (Figure 1-3).

Figure 1-5: *FoxP1* mRNA expression in Area X of adult male Bengalese finches.



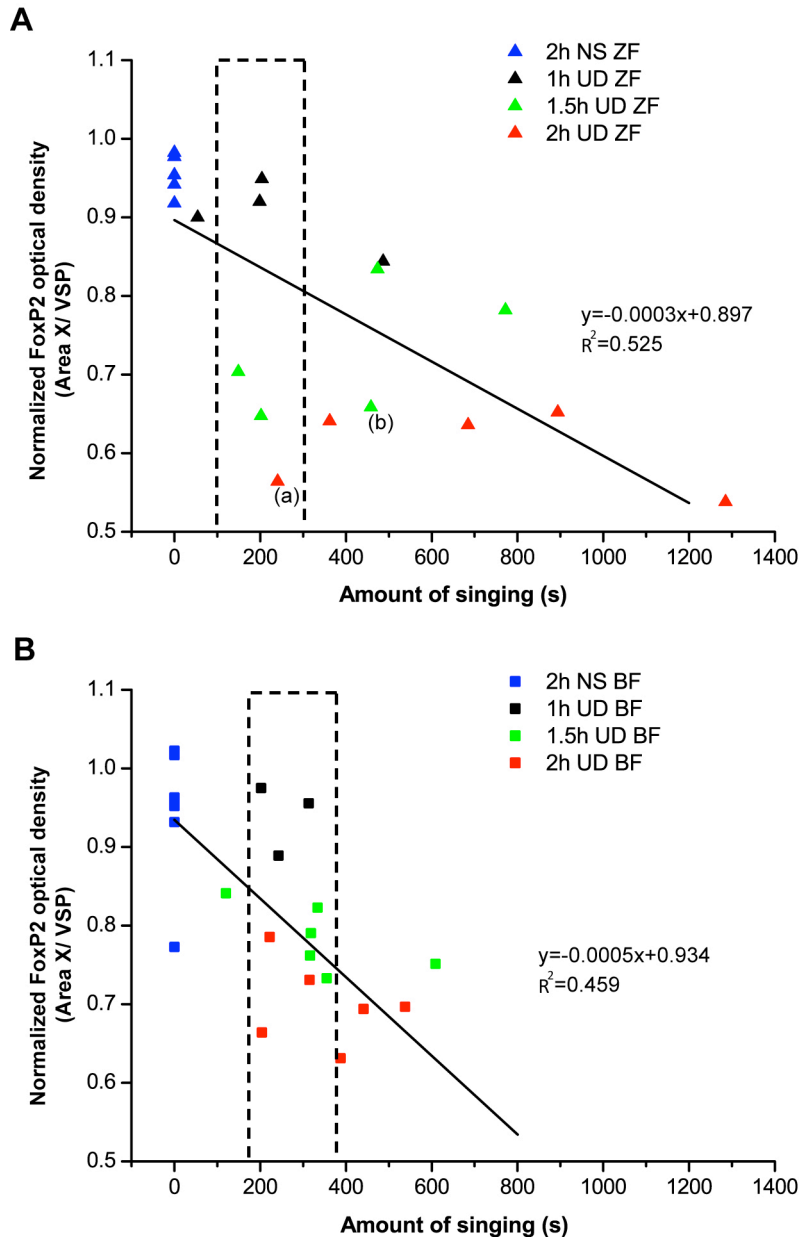
A) Bright-field photomicrograph of Nissl-stained hemi-coronal section with schematic drawing highlights song nuclei LMAN and Area X. B) Representative images of *FoxP1* mRNA expression at the level of Area X in 2h NS (left) and 2h UD (right) adult male Bengalese finch brain. There is no apparent effect of singing on expression levels. Location of sections corresponds to the level of transverse plate 11 in the zebra finch atlas of Nixdorf-Bergweiler and Bischof (2007). Scale bar 1mm. D, dorsal; L, lateral.

Figure 1-6: *FoxP2* mRNA expression within Area X diminishes after birds sing undirected songs.



A) Top, schematic drawing based on a Nissl stained hemi-coronal section is shown together with a control hemi-section incubated with sense RNA. Beneath, representative bright-field photomicrographs of *FoxP2* mRNA expression patterns in hemi-coronal sections from Bengalese finches (left) and zebra finches (right) of different behavioral groups are shown together with corresponding Nissl-stained hemi-sections. Scale bar 1mm. D, dorsal; L, lateral. B) Quantitative results of *FoxP2* mRNA expression level within Area X relative to VSP. Box indicates s.e.m.. Dots in boxes indicate mean. Whiskers indicate max and min. 2h NS BF: n=7; 1h UD BF: n=3; 1.5h UD BF: n=6; 2h UD BF: n=6; 2h NS ZF: n=5; 1h UD ZF: n=4; 1.5h UD ZF: n=5; 2h UD ZF: n=6. Kruskal–Wallis nonparametric ANOVA, * p<0.001.

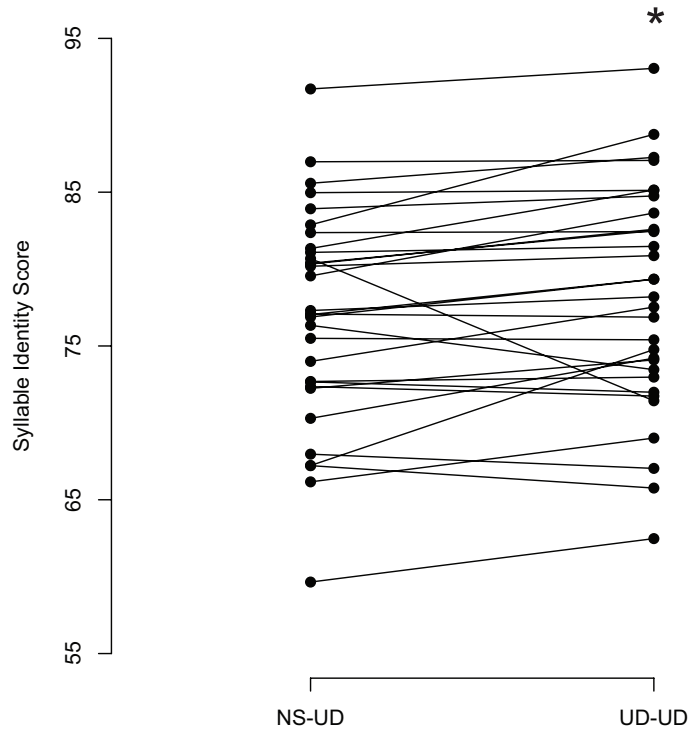
Figure 1-7: Correlation between *FoxP2* and amount of singing.



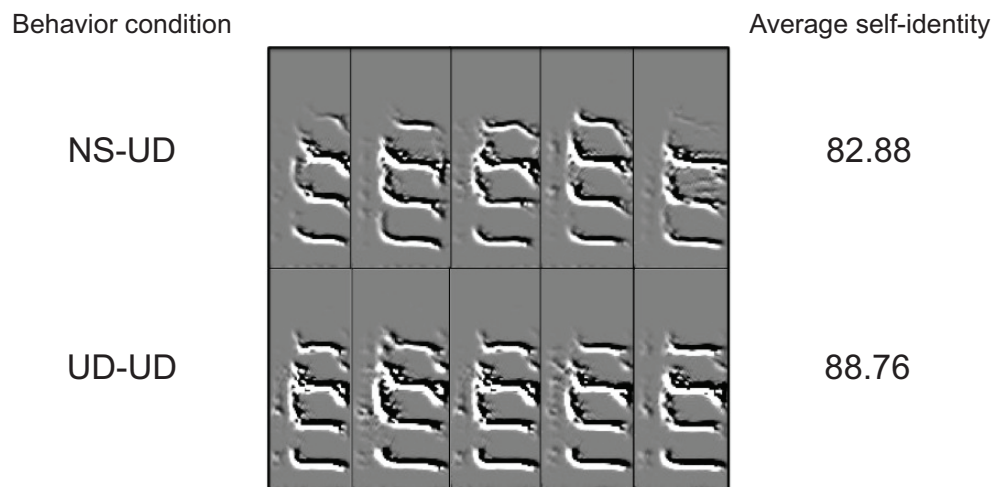
A) In zebra finches, *FoxP2* levels decrease as the amount of singing increases ($p < 0.0002$) B) *FoxP2* levels also decrease as the amount of singing increases in Bengalese finches ($p < 0.0003$) There is no significant difference between the two regression lines ($p > 0.05$). The dotted rectangle indicates data from those birds that sang similar amounts of song for each species (see Discussion).

Figure 1-8: Behavioral changes in syllable self-identity.

A



B



A) Paired plot of syllable cluster self-identity in NS-UD (left) and UD-UD (right) conditions. Denoted by an asterisk, the UD-UD condition had higher mean self-identity ($p=0.034$, 2-tailed paired bootstrap). B) Representative spectral derivatives of five syllables from one cluster in the NS-UD (top) and UD-UD (bottom) conditions with self-identity scores reported (right).

Tables

Table 1-1: Mean optical density (OD) values measured from multiple sections of different groups of Bengalese finches.

Brain region	<i>FoxP1</i>			<i>FoxP2</i>		
	UD Male	NS Male	Female	UD Male	NS Male	Female
A	0.43	0.57	0.46	0.57	0.56	0.8
Area X	1.41	1.31	N/A	1.94	2.7	N/A
Bas	0.62	0.68	0.54	0.72	0.89	1.02
Dorsal thalamus	1.05	1.17	1.02	3.47	4.19	4.41
HA	0.91	0.99	0.95	1.01	0.97	0.89
HD	1.45	1.4	1.22	1.19	1.12	1.21
HVC	1.31	1.24	N/A	0.99	1.03	N/A
LMAN	0.59	0.73	0.54	0.92	1.07	1.02
M	1.45	1.4	1.23	1.24	1.2	1.29
N	1	1	1	1	1	1
RA	0.58	0.71	N/A	0.62	0.57	N/A
Striato-pallidum	1.3	1.3	1.17	2.42	2.48	2.74

All values are normalized to the mean value of the OD in nidopallium outside of song control areas.

Table 1-2: The average CV and standard deviation (SD) for each acoustic feature

Acoustic feature	NS-UD	SD	UD-UD	SD	p-value
	Mean Values				
Pitch	0.29	0.251	0.282	0.272	0.7169
Frequency Modulation	0.147	0.068	0.136	0.058	0.1242
Entropy	0.127	0.053	0.12	0.055	0.0376
Pitch Goodness	0.226	0.121	0.193	0.11	0.0002
Mean Frequency	0.166	0.081	0.151	0.078	0.0168
	Variance				
Frequency Modulation	0.206	0.069	0.198	0.087	0.38
Entropy	0.5	0.259	0.44	0.184	0.0136
Pitch Goodness	0.662	0.319	0.581	0.412	0.1078
Mean Frequency	0.895	0.678	0.775	0.328	0.1901

The average CV and SD for all syllable clusters within each behavioral condition is reported along with p-values generated by a 2-tailed paired bootstrap test. Significant values are shown in bold font.

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Chapter 2: Attenuation of Cntnap2 Expression with shRNA constructs

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Abstract

Mutations in contactin associated protein-like 2 (Cntnap2) are associated with cortical dysplasia-focal epilepsy, autism spectrum disorder, and specific language impairment, among other disorders. Rodent models can be used to study the impact Cntnap2 has on many symptoms of autism, such as social impairments, repetitive behaviors, and altered sensitivity to stimulation. However, one other common deficit, language impairment, may be better studied in an animal model of vocal learning. Songbirds such as the zebra finch (*Taeniopygia guttata*) are among the few animal groups outside of humans that have proven vocal learning. The neural circuitry underlying vocal learning is similar between humans and zebra finches. A major experimental advantage of the songbird model is that these brain regions responsible for vocal learning are organized into anatomically distinct structures called song control nuclei, within which neurons are dedicated to vocal learning and production. We have previously characterized the expression of Cntnap2 in the zebra finch song system. To further test the function of Cntnap2, we developed shRNA constructs to specifically knock down zebra finch Cntnap2 and confirmed the knockdown effect in human embryonic kidney cell line and zebra finch primary telencephalic cultures. The shRNA constructs were then put into an adeno-associated virus (AAV) to serve as a vehicle for future *in vivo* transgene expression.

Introduction

FOXP2 is linked to neural pathways underlying human speech and language. As a transcription factor, FOXP2's effects on language must be mediated through its gene targets. *CNTNAP2* is one such target; chromatin-immunoprecipitation studies reveal that the transcription factor binds within the first *CNTNAP2* intron and thereby represses its expression. Accordingly, *CNTNAP2* and *FOXP2* exhibit inverse expression patterns across human cortical layers, with high *CNTNAP2* and low *FOXP2* levels observed in layers II and III of the cortical plate (Vargha-Khadem et al., 2005; Vernes et al., 2008; 2011). *CNTNAP2* has independently been associated with a language-related disorder. A group of Old Order Amish children who exhibited seizures, autistic features, and language regression, was discovered to harbor a loss-of-function mutation in *CNTNAP2* (Strauss et al., 2006). Within the general population, *CNTNAP2* polymorphisms are associated with language-related disorders, including increased risk for autism spectrum disorder (ASD) (Arking et al., 2008; Bakkaloglu et al., 2008; Li et al., 2010), delayed age of first word (Alarcón et al., 2008) and specific language impairment (SLI) (Newbury et al., 2011; Peter et al., 2011; Whitehouse et al., 2011). Moreover, brain imaging reveals that carriers of the *CNTNAP2* risk allele exhibit decreased long-range connectivity of the medial prefrontal cortex, decreased lateralization (Scott-Van Zeeland et al., 2010) and reduced frontal gray matter (Tan et al., 2010). *CNTNAP2* variants are also associated with detectable structural connectivity in young adults (Dennis et al., 2011).

The mechanism whereby *CNTNAP2* affects speech and language remains unclear. *CNTNAP2* is a member of the neurexin super-family. Polymorphisms of other neurexins and their synaptic binding partners, neuroligins, are implicated in ASD and hypothesized to contribute to cognitive disorders by compromising synaptic development (Kwon et al., 2012;

Schmeisser et al., 2012). The most well-characterized function of *Cntnap2* is to organize the nodal microdomain of myelinated axons in the peripheral nervous system (Poliak et al., 2003). The *Cntnap2* gene encodes a membrane protein that localizes voltage-gated potassium channels (VGKCs) to the nodes of Ranvier along axons (Poliak et al., 2003; Horresh et al., 2008). Evidence from mouse neuronal cultures indicates that *Cntnap2* has an earlier organizational function in developing neurons before myelination occurs, as follows: *Cntnap2* affects synapse formation and neural network assembly indirectly by controlling the stabilization of new dendritic spines (Anderson et al., 2012; Gdalyahu et al., 2015).

Transgenic mice lacking *Cntnap2* exhibit behavioral abnormalities reminiscent of ASD, such as epilepsy, hyperactivity, diminished social activity, repetitive behaviors, and reduced frequency of ultrasonic vocalizations when pups are separated from their dams (Peñagarikano et al., 2011). Rodent models of ASD provide invaluable tools for investigation of certain aspects of the disorder. However, the learned vocal communication component of ASD is not fully addressable with these models because they have largely innate vocal communication signals (Kikusui et al., 2011; Arriaga et al., 2012; Day and Fraley, 2013). Cross-species comparisons among mammals show that the brain distribution of the mRNA encoding *Cntnap2* differs between vocal learners and non-vocal learners: it is enhanced in language-related cortico-basal ganglia circuitry in human embryos, in contrast to a broad, even distribution in mouse or rat embryos (Abrahams and Geschwind, 2008).

Unlike rodents, songbirds learn a portion of their vocalizations in a manner that shares significant similarities to human speech learning. Like human speech, birdsong: 1) occurs spontaneously within the organism's species-characteristic behavior; 2) is learned through social interactions with conspecifics; 3) happens during defined developmental critical periods; 4)

responds to hormonal experience, 5) relies on auditory experience, 6) compensates for experimentally imposed errors, 6) relies on dedicated cortico-basal ganglia circuitry, and 7) exhibits hemispheric lateralization (Wade and Arnold, 2004; Wang et al., 2008; Brainard and Doupe, 2013). Furthermore, recent transcriptomic studies indicate that vocal control regions of the human and zebra finch brain share greater similarity in gene expression profiles than do the corresponding regions of non-human primates, and non-vocal learning birds, respectively (Pfenning et al., 2014). Uniquely among lab animal models, the loci for vocal learning are neuroanatomically distinct and have been well characterized in sexually dimorphic songbird species such as zebra finches (White, 2010). Thus, the learned song of the zebra finch can provide much-needed insights into ASD speech and social deficits to complement findings from rodent studies. Elucidating the function of *Cntnap2* in songbirds may help to better understand developmental delays in speech and language acquisition associated with certain ASD endophenotypes.

In order to study the function of *Cntnap2* in songbirds, we needed to develop a tool to modify the expression of the zebra finch *Cntnap2* gene. Though generating transgenic zebra finches has been proved technically possible (Agate et al., 2009; Abe et al., 2015), using a virally-mediated methodology to knock down *Cntnap2* is still a better choice. Production of transgenic zebra finches is laborious and inefficient. There are no proven methods for restricting expression to specific neuronal subtypes. Therefore, transgene could have off-target effects. While globally modifying *Cntnap2* in zebra finch can be interesting, it can be difficult to isolate the specific effects on vocal learning behavior. Transgenic repression of *Cntnap2* in zebra finches may result in non-vocal behavioral effects that could confound effects on vocal learning.

Here, we developed shRNA constructs to specifically knock down zebra finch *Cntnap2*.

The pCIGRNAi vectors (Figure 2-1A) tested here had been previously effective in knockdown experiments on chicken embryos (Megason and McMahon, 2002). The vectors include a chicken U6 promoter to express RNAs modeled on microRNA30, which are embedded within chicken microRNA operon sequences to ensure optimal Drosha and Dicer processing of transcripts. Perhaps not surprisingly, the chicken U6 promoter works significantly better in chick embryos than promoters of mammalian origin and, in combination with a microRNA operon expression cassette (MOEC, Figure 2-1B), achieves up to 90% silencing of target genes.

After the knockdown effect of designed shRNAs has been confirmed, a viral vector is used to deliver the shRNAs *in vivo* into zebra finch brains. Lentiviruses have been successfully used to deliver RNAi into zebra finch song control nuclei in the past (Wada et al., 2006; Haesler et al., 2007), but we have chosen instead to use AAV. This latter virus was used previously to overexpress FoxP2 in zebra finch song nuclei (Heston and White, 2015). AAV can also express a transgene for more than 6 months, with less risk of random insertion into the genome, which potentially causes oncogenesis (Papale et al., 2009; Doherty et al., 2011). Though less genetic material can be inserted into the backbone of an AAV than a lentivirus, the relatively small amount required for shRNA expression is not a challenge. There are several AAV serotypes, each of which has different tropisms for specific cell types. AAV serotype 1 exhibits a high tropism for neurons providing optimal transduction efficiency in the brain. Thus, the MOEC from pCIGRNAi was cut out and inserted into the AAV1 backbone. Within the same construct, the reporter gene, green fluorescent protein (GFP), driven by chicken β -actin (CBA) promoter served as a tag (Figure 2-1C).

Materials and Methods

RNAi design

Three short interfering RNA (siRNA) constructs were designed using the BLOCK-iT™ RNAi designer tool. In order to ensure that both splice variants of *Cntnap2* would be targeted by the same construct, only the part of the zebra finch *Cntnap2* cDNA (NM_001193337) corresponding to the last 140 amino acids of the resulting C termini of the two protein isoforms (nucleotides 4104-4524) was targeted. This region encompasses part of the extracellular domain between the first laminin-G domain and the transmembrane region itself (Figure 2-2). These regions were included for targeting because they were conservative. The alternative splicing of closely-related neurexin-1, which includes a single laminin-G domain and the homologous transmembrane region (Ushkaryov et al., 1994; Kleiderlein et al., 1998). The three 19mer siRNA designs rated highest by the BLOCK-iT™ design tool were chosen for further processing. Each design was named for its corresponding start position in the zebra finch *Cntnap2* cDNA: siRNA-4282, -4288, -4372. The following criteria for maintaining each siRNA as a candidate knockdown sequence were: 1) no more than 1 mismatch with the zebra finch *Cntnap2* genomic sequence, 2) no less than 2 mismatches with any transcript in either the zebra finch genome or EST libraries, 3) no less than 2 mismatches with any transcript in the human genome or EST libraries. The last criterion was included so that the siRNAs could be tested against zebra finch *Cntnap2* overexpressed in a human embryonic kidney (HEK 293) established cell line. Since the HEK 293 cell line demonstrated no endogenous *CNTNAP2* expression, homology between the candidates and human *CNTNAP2* was not included as a criterion, though a BLAST search failed to match the candidates to the human sequence. All three candidates met the first two criteria. These three sequences matched the genomic zebra finch *Cntnap2* sequence, except siRNA-4372,

for which the 19th base was a T in the designed siRNA and a C in the corresponding genomic position. siRNA duplexes with fluorescein tags on the 5' end of the guide strand were purchased from Life Technologies (Carlsbad, CA). 19mer siRNA sequences were modified to 21mer shRNA through the BLOCK-iT RNAi designer by restricting the input target sequence from the zebra finch *Cntnap2* cDNA to 100bp surrounding the 4282, 4288, and 4372 target sites. An additional shRNA, 4328, was generated using the same algorithm, but using nucleotides 4104-4524 of the zebra finch *Cntnap2* cDNA as an input. Each 21mer was again modified to a 22mer by adding the nucleotide to the 5' end that corresponds to the target site, as in (Dow et al., 2012). A 22mer non-targeting shRNA, which does not target either zebra finch *Cntnap2* or any other genes in zebra finch genome for more than 13 nucleotides was designed as the control shRNA, and named 'shGEN'. Table 2-1 shows the target sequences of all the designed shRNAs including shGEN.

shRNA constructs

pCIGRNAi empty vector (Megason and McMahon, 2002; Skaggs et al., 2011) was obtained as a gift from Bennett Novitch. The procedure for cloning shRNAs into the vector were adapted from (Van Hateren et al., 2009) and are briefly described here. All primers described in the following section were obtained from Life Technologies. For adding shRNA into the two cloning sites of pCIGRNAi, specific forward and reverse primers consisted of each shRNA target sequence with or without a mismatched nucleotide on the 5' end (A switched to C, T switched to G, and vice versa).

For the first cloning site of pCIGRNAi, the gene specific primers were then designed as follows: forward 5'-AGGTGCTGCCAGTGAGCG <insert target sequence with mismatch> TAGTGAAGCCACAGATGTA-3'; reverse 5'-CACCACCACCAGTAGGCA <insert target sequence> TACATCTGTGGCTTCACT-3'. For the second cloning site of pCIGRNAi, the gene specific primers were designed as follows: forward 5'-GTTCTCCGCAGTGAGCG <insert target sequence with mismatch> TAGTGAAGCCACAGATGTA 3'; reverse 5'-GAAGACCAGCAGTAGGCA <insert target sequence> TACATCTGTGGCTTCACT 3'. Oligonucleotides for insertion into the pCIGRNAi vector were constructed by polymerase chain reaction (PCR). For each shRNA, 200ng each specific forward and reverse primer was combined with 2µL each of 10µM universal forward and reverse primers (1st cloning site forward U1F 5'-GGCGGGGCTAGCTGGAGAAGATGCCTTCCGGAGAGGTGCTGCCAGTGAGCG-3'; 1st cloning site reverse U1R 5'-GGGTGGACGCGTAAGAGGGGAAGAAAGCTTCTAACCCCGCTATTCACCACCACCAGTAGGCA-3'; 2nd cloning site forward U2F 5'-GGCGGGACGCGTGCTGTGAAGATCCGAAGATGCCTTGCGCTGGTTCCTCCGCAGTGA GCG-3'; 2nd cloning site reverse U2R 5'-CGCCGCGCATGCACCAAGCAGAGCAGCCTGAAGACCAGCAGTAGGCA-3'), 25µL GoTaq® Green Mastermix (Promega, Madison, WI, Cat. No. M712B), and nuclease-free water added for a final volume of 50µL. Each reaction was initially heated to 95°C for 2 minutes, then subjected to 40 cycles of melting at 95°C for 30s, annealing at 55°C for 30s, and elongating at 72°C for 60s. After the last cycle, the reaction was finished with a final elongation step for 4 min. The entire PCR reaction was resolved on a 2% agarose gel, and the ~180bp band was extracted using a gel extraction kit (PureLink™, Life Technologies, Cat. No. K2100-12). For cloning shRNAs into the first cloning site, the entire elution was digested in a 50µL reaction with one

unit each FastDigest *NheI* and *MluI* (Thermo Scientific, Waltham, MA, Cat. No. FD0974 and FD0564, respectively). PCR digests were again resolved on a 2% agarose gel and the ~180bp band extracted. pCIGRNAi vector was linearized by digestion with one unit each of *NheI* and *MluI*, then treated with calf intestinal alkaline phosphatase (Life Technologies, Cat. No. 18009-027), and purified with a PCR purification kit (PureLink™, Life Technologies, Cat. No. K3100-01). For cloning shRNAs into the second cloning site, *SphI* (Thermo Scientific, Waltham, MA, Cat. No. FD0604) was used instead of *NheI*. 4ng of PCR digest was combined with 50ng linearized pCIGRNAi plasmid, and ligated with 1μL T4 DNA ligase (Life Technologies, Cat. No. 15224-017), at room temperature for 2h. Chemically competent *E. coli* (One Shot® Mach1™T1, Life Technologies, Cat. No. C8620-03) were transformed with 5μL of the ligation reaction and grown on agar plates with 50mg/mL ampicillin overnight.

Individual bacterial colonies were set up to grow in liquid culture. PCR screening was used to select the successful cloning of shRNAs. To do so, 1ul of bacterial solution was mixed with screening primers 0.25μg of screening primers (V1: 5'-TCCCGGCTCGGGGCAGCTTC-3', 1st cloning site: V1+U1R; 2nd cloning site: V1+U2R), 25μl GoTaq® Green Mastermix and nuclease-free water for a final volume of 50μL. PCR was carried out as previously described. The entire PCR reaction was resolved on a 2% agarose gel; the positive colony for first cloning site inserts should have a 200bp band while the positive colony for second cloning site inserts should have a 300bp band. Plasmid DNA from the positive colonies was extracted by maxiprep (Plasmid *Plus* Maxi kit, Qiagen, Cat. No. 12963) and sequenced by the UCLA Genotyping and Sequencing Core using a chicken U6 primer (ACAGTCACTGTGTTCTAAAAGAACTTG) to verify insertion of the shRNA constructs.

HEK 293 cell culture

HEK 293 cells were received as a gift from Professor Kelsey Martin at UCLA in the form of a 1mL culture frozen in liquid nitrogen. The culture was rapidly warmed to 37°C in a water bath, then added to 9mL of prewarmed 37°C HEK cell culture medium, comprised of Dulbecco's modified eagle medium (DMEM; Life Technologies, Cat. No. 11995-065) with 10% fetal bovine serum (FBS; Life Technologies, Cat. No. 10082-139) and 1% Antibiotic-Antimycotic solution (Life Technologies, Cat. No. 15240-062). Cell suspension was centrifuged at 500rcf for 5 minutes, then the supernatant was discarded, leaving a cell pellet. Cells were resuspended in fresh culture medium, and then plated in 10cm petri dishes (Fisher Scientific, Waltham, MA; Cat. No. 353003) and incubated at 37°C with 5% CO₂. Cells were passaged as needed to keep the density on the plate below confluence by sucking up culture medium in a Pasteur pipette and releasing it to apply pressure to dissociate cells from the plate. Cells were then re-plated at a higher dilution, sometimes onto multiple plates. Aliquots of HEK cultures were frozen by dissociating cells from a nearly confluent plate, centrifuging at 500rcf for 5 min, reconstituting cells in 750µL culture medium, then adding an equal volume of culture medium with 20% dimethyl sulfoxide (DMSO; Fisher Scientific, Cat. No. BP231-100) dropwise while vigorously mixing. The cell suspension in 10% DMSO was immediately frozen to -20°C for 2-4 hours before freezing to -80°C for 16-24 hr. Frozen cultures were permanently stored in liquid nitrogen.

Plasmid transfection into HEK 293 cells

pCIGRNAi plasmids with *Cntnap2* shRNA inserts were co-transfected with a pCDNA3.1 vector expressing the coding sequence of zebra finch *Cntnap2* (Accession number NM_001193337.1) into HEK 293 cells. The day before transfection, HEK 293 cells were plated to ~10% confluence in a 10cm plate. The morning of transfection, the culture media was removed and replaced with 8mL fresh media. Aliquots of 500 μ L HEPES buffered saline were made for each transfection. 20 μ g of total DNA was added to each aliquot and mixed well before adding 37.5 μ L of 2M calcium chloride. The transfection solution was incubated at room temperature for 20 min, and then added dropwise to the appropriate plate. In some of the experiments, 6-well plates were used instead of 10cm plates. The amount of solution and plasmids used was adjusted accordingly. 16h after transfection the media was changed and cells were cultured for another 24h before protein was harvested as described below.

Western blot analysis

Cell culture medium was removed from the dish, cells were washed once in phosphate buffered saline, then incubated on ice in radioimmunoprecipitation assay (RIPA) lysis buffer with 10% protease inhibitor cocktail (Sigma Aldrich, Cat. No. P8340) for 20 minutes. Lysates were collected and stored at -80°C. To determine the concentration of total protein, a Lowry assay was performed using an RC-DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, Cat. No. 500-0120). 25 μ g protein was diluted into 25 μ L RIPA lysis buffer, then added to an equal volume of 5% beta-mercaptoethanol (Sigma Aldrich, Cat. No. M6250) in Laemmli buffer (Bio-Rad, Cat. No. 161-0737). Samples were boiled for 2 minutes, and then resolved on a 10% isocratic SDS-polyacrylamide gel in tris-glycine-SDS buffer (Bio-Rad) at 200V. A Precision

Plus Protein™ Dual Color Standard (Bio-Rad) was included on the gel as a molecular mass marker. Protein was then transferred onto a PVDF membrane with a pore size of 0.45µm in tris-glycine (Bio-Rad) with 20% methanol and 1% SDS. The membrane was stained with Ponceau S solution (Sigma Aldrich) and cut along the 50 kD molecular mass marker. Both halves of the membrane were blocked with 5% milk in tris-buffered saline with 0.1% tween-20 (TBST) for 1 hour. Subsequently the half containing proteins >50kD was incubated in anti-Cntnap2 antibody (Millipore) solution diluted 1:2000 in 2.5% milk-TBST overnight at 4°C. The half containing proteins <50kD was incubated in anti-GAPDH antibody (Millipore, Cat. No. MAB374) diluted at either 1:100,000, 1:50,000, or 1:20,000 in 2.5% milk-TBST overnight at 4°C. The blot was washed in TBST, then incubated in HRP-labeled secondary antibodies (GE Healthcare, Piscataway, NJ) diluted in 2.5% milk-TBST for 2 hours: >50kD in anti-rabbit diluted to 1:2000, <50kD in anti-mouse diluted to 1:10,000. The blot was washed in TBST, then developed with ECL Plus and imaged on a Typhoon scanner (GE Healthcare) and signal specificity assessed.

Zebra finch primary telencephalic culture

L5-5 culture medium was prepared from Minimal Essential Medium with Earle's and Glutamax (MEM; Invitrogen, Cat. No. 41090-036), supplemented with 1:40 fetal bovine serum (Hyclone Cat. No. SH30070.02), 1:40 horse serum (Gibco, Cat. No. 26050-070), 1:100 30% glucose, 1:100 penicillin/streptomycin mixture (Invitrogen, Cat. No. 15070-063), 1:300 insulin transferrin sodium selenite (Sigma, Cat. No. I1884), and 1:50 B27 (Invitrogen Cat. No. 17504-044). All components were mixed and passed through a 0.2 µm filter.

1-2 hatchling zebra finches 1-4 days old were rapidly decapitated, telencephali quickly extracted and stored temporarily in Leibovitz's L-15 medium (Fisher Scientific, Cat. No. 21083-027) on ice. The hemispheres were separated with a scalpel blade, then the meninges carefully removed with forceps. Each hemisphere was bisected, then transferred to a 15mL conical tube and excess media was aspirated. Cells were digested with 0.5% papain (Sigma Aldrich, St. Louis, MO, Cat. No. P-4762), 0.03% bovine serum albumin (BSA; Sigma Aldrich, Cat. No. A7030) in L-15 medium heated to 37°C for 15 minutes. Cells were dissociated in L5-5 medium by triturating with glass pipettes with increasingly smaller diameters for 15 minutes. Solution containing the dissociated cells was strained with a 70µm strainer. The density of dissociated cells in medium was estimated by adding 10µL trypan blue (Sigma Aldrich, Cat. No. T8154) to an equal volume of cell suspension and counting on a hemacytometer. The culture was then incubated at 37°C and 5.0% CO₂.

Plasmids transfection into zebra finch primary telencephalic culture

Cells were cultured in six-well plates (Fisher Scientific, Cat. No. 08-772-1B) on poly-D-lysine/laminin coated glass coverslips (Fisher Scientific, Cat. No. 08-774-385), with two coverslips per well. 2-3 million cells were cultured in each well. pCIGRNAi plasmids with shRNA or shGEN inserts were transfected with Lipofectamine LTX Plus reagent (Life Technologies, Cat. No. A12621). Transfection was performed 18h after plating. 2µg DNA was mixed with 6µl LTX, 2µl PLUS and 250µl MEM to form the transfection solution. The transfection solution was incubated at room temperature for 5 min, and then added dropwise to the appropriate well.

Viral transduction

AAVs with a CAG promoter driving expression of GFP and a chicken U6 promoter driving the MOEC with 4328+4372 shRNAs or shGEN were ordered from Virovek (Hayward, CA). Both AAVs are $\sim 2 \times 10^{13}$ vg/ml. 25 μ l aliquots were made and stored at -80°C before use. In all experiments, viral transduction was performed at 5 days after plating (DIV5), and the virus was removed 14-16 hours after transduction by changing the L5-5 culture medium. 3.0 μ l of virus was added to each well during transduction.

Immunocytochemistry

Since cultures contained GFP, all subsequent steps were performed in low light conditions. Coverslips of telencephalic cultures were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 min. and then washed three times with tris-buffered saline (TBS) to remove paraformaldehyde. Cells were blocked with 10% goat serum in TBS for 1hr, then incubated overnight at 4°C with anti-Cntnap2 antibody (anti-Caspr2; Millipore, Temecula, CA, Cat. No. AB5886) diluted at 1:2,000 in TBS with 1% goat serum. Primary antibody was washed away with TBS, then cells were incubated in goat-anti-rabbit IgG Alexa Fluor® 555 secondary antibody (Life Technologies, Cat. No. A11010) diluted at 1:1,000 in TBS with 1% goat serum for 2hr. Coverslips were mounted onto slides using Prolong Antifade Gold Reagent (Life Technologies, Cat. No. P36934) and stored at 4°C until use.

Image acquisition and analysis

Images were acquired using an Axio Imager.A1, with an AxioCam HRm digital camera (Carl Zeiss Inc., Oberkochen, GE). Axiovision software (Carl Zeiss Inc.) was used to optimize photomicrographs to remove background, improve brightness and contrast, and to pseudocolor the images. Cntnap2 is represented here in red; GFP is represented in green. Adjustments were made to the entire image and not to selective subregions.

Quantification of Cntnap2 knockdown was performed in both plasmid-transfected and AAV transduced cells. Exposure time for the red channel during picture acquisition was reduced to the threshold at which barely any red signal was detected in GFP+ cells in shRNA groups. Pictures of the shRNA groups and shGEN control group were then taken at the same exposure time. Total number of GFP+ cells, as well as Cntnap2 and GFP+ cells were manually counted by two observers independently.

Results

The 4282, 4288, 4328 and 4372 shRNA constructs were designed to target the 3' end of the zebra finch *Cntnap2* coding sequence (Figure 2-2). The rationale for designing constructs to target the 3' end of the coding sequence was a concern over potential *Cntnap2* splice variants. Two splice variants have been identified in mouse (Poliak et al., 1999), though little has been reported regarding the shorter β isoform. The murine *Cntnap2\beta* (NM_025771.3) retains the 3,279 nucleotides on the 3' end of the coding sequence of the full-length α isoform (NM_001004357.2), including the region that codes for the transmembrane region and the first laminin G domain (Figure 2-2). Therefore, shRNAs were designed to target only this region of the zebra finch isoform of *Cntnap2* (Condro's thesis Chapter 3).

As previously shown (Michael Condro's UCLA thesis Figure 3-3), when 4282, 4288 and 4372 siRNA were co-transfected into HEK 293 cells along with a plasmid expressing zebra finch *Cntnap2*, all 3 siRNAs reduced *Cntnap2* protein expression relative to the scrambled control. siRNA duplexes allow direct interference with the mRNA in transfected cells without the intermediate step of transcription from a plasmid vector. However, the tradeoff is that the effect is transient, often lasting no longer than 1-2 days (Strapps et al., 2010). In order to obtain stable knockdown effect, we inserted 4282, 4288 and 4372 along with the untested 4328 shRNA into the plasmid pCIGRNAi. The corresponding sequences were cloned into the first cloning site of pCIGRNAi. Four single-shRNA constructs were then co-transfected into HEK 293 cells along with plasmid expressing full-length zebra finch *Cntnap2* at a 9:1 ratio. To determine the extent of *Cntnap2* expression in the absence of knockdown constructs, one well was transfected with zebra finch *Cntnap2* and empty pCIGRNAi vector, serving as a 'positive control'. *Cntnap2* expression from each of the other wells was normalized against the positive control levels. Another well

received transfection reagents alone, without any plasmid and thus served as a negative control. 70% reduction in protein is considered effective knockdown (Ui-Tei and Saigo, 2004). By this standard, plasmids containing 4372 and 4328 successfully attenuated *Cntnap2* expression in HEK 293 cell cultures (Figure 2-3). To test for even greater knockdown, a given culture was simultaneously exposed to a pair of shRNA constructs; all six different combinations of two single-shRNA constructs were tested. shRNA pairs were co-transfected with zebra finch *Cntnap2* at 4.5:4.5:1 ratio. *Cntnap2* expression was normalized to the positive control. The combinations of “4282, 4288”, “4372, 4328”, and “4282, 4328” exhibited the greatest knockdown effect (Figure 2-4).

The corresponding shRNAs were cloned into the second cloning site of pCIGRNAi to obtain three double-shRNA constructs: 4328+4288, 4328+4372 and 4282+4288. The non-targeting control construct shGEN was also generated with the same method with shGEN sequence inserted in the first cloning site of pCIGRNAi. Three double-shRNA constructs were then co-transfected into HEK 293 cells with zebra finch *Cntnap2* at a 9:1 ratio. The three combinations of single-shRNA were retested at the same time in other wells. Positive and negative controls were set up as described above. *Cntnap2* expression was normalized to relative to positive control levels. The double-shRNA constructs reduced *Cntnap2* expression by >95%, indicating even greater effectiveness than the combinations of single-shRNA constructs (Figure 2-5). The non-targeting control shGEN did not have a significant knock down effect on *Cntnap2*.

We have evaluated the use of shRNAs targeting *Cntnap2* cDNA in HEK cell culture, but this may not be an accurate reflection of the ability of these constructs to reduce endogenously expressed *Cntnap2* in zebra finch cells. Possible reasons include mismatches between the cDNA and genomic versions of the gene or potentially toxic off-target effects of the shRNA in zebra

finch tissues. Therefore, double-shRNA constructs were transfected into zebra finch primary neuronal cultures, which express endogenous Cntnap2. shGEN construct and pCIGRNAi were also tested as controls. Cells were fixed at DIV4 and conventional immunocytochemistry was used to identify Cntnap2 expression. The experiment was repeated once and the replicate results for the same conditions were aggregated. The total number of GFP+ cells as well as Cntnap2 & GFP+ cells was counted in each group. Two observers did the counting independently. Average numbers were shown in Table 2-2. Due to technical issues, the well receiving 4328+4288 had a relatively small number of GFP+ cells. The total number of Cntnap2 and GFP+ cells were counted and divided by the number of GFP+ cells to determine the Cntnap2 expression level in primary cultures. The percentage of Cntnap2+ cells was significantly smaller in cultures, which received the double-shRNA constructs, compared to the ones that received the shGEN or pCIGRNAi (Figure 2-6, χ^2 test, for “4328+4288”, $p < 0.005$, for others, $p < 0.0001$).

Taking into consideration the results from both the HEK cells and primary cultures, all three of double-shRNA constructs successfully knocked down zebra finch Cntnap2. We decided upon 4328+4372 to continue with further experiments, namely, the development of an AAV suitable for in vivo delivery and knock down. The 4328+4372 and shGEN constructs were sent to Virovek for commercial preparation. The resultant viruses, AAV1-CBA-GFP-MOEC-4328-4372 (“AAV-KD”) and AAV1-CBA-GFP-MOEC-shGEN (“AAV-shGEN”) were provided by the company.

Both AAV-KD and AAV-shGEN were tested in zebra finch primary cultures. The AAV-KD was found to be effective in knocking down Cntnap2 (Figure 2-7, Table 2-3). The AAV-KD-transfected cultures contained a significantly smaller percentage of cells expressing Cntnap2 compared to those transfected with AAV-shGEN control at one day (χ^2 test, $p = 1.37E-$

05<0.0001) and four days after transfection (χ^2 test, $p = 0.003 < 0.005$). At one week after transfection, this increase was no longer significant (χ^2 test, $p = 0.462$). Based on the effectiveness of this construct *in vitro* in HEK cells and zebra finch primary cultures, via delivery of naked shRNA, plasmid or virus, we proceeded to use the AAV-KD and AAV-shGEN for *in vivo* studies described in Chapter 3.

Discussion

The long-term goal of this study is to attenuate *Cntnap2* expression *in vivo* within the zebra finch song control system to determine *Cntnap2*'s contribution to vocal production and song learning. Thus, finding a tool to successfully knockdown zebra finch *Cntnap2* is a crucial step. Here, four shRNAs were shown to successfully attenuate the expression of *Cntnap2* in HEK293 cell cultures (Figure 2-3). The combinations of shRNAs exhibited a greater knockdown effect (Figure 2-4, Figure 2-5) than that obtained with any single shRNA. The ability of these shRNAs to knockdown endogenous zebra finch *Cntnap2* was further confirmed in zebra finch telencephalic cultures (Figure 2-6). Based on these results, a pair of shRNAs was chosen and inserted into AAV backbone to create AAV-KD. When applied to zebra finch cultures, the AAV-KD decreased *Cntnap2* signals relative to cultures that received the control virus, but only in the short term. A significant knockdown effect was not found at one week after transfection. This is likely due to the small DIV12 sample size compared to the DIV6 and DIV9 groups (Table 2-3). A significant effect may be found if a larger sample size is used.

The success of this AAV in knocking down zebra finch *Cntnap2* in culture bodes well for its use *in vivo*. Stereotaxic targeting of the construct specifically to song control nuclei and precisely at the critical periods for song learning would allow us to assess the role of *Cntnap2* in vocal learning. Moreover, underlying changes in neuronal morphology and connectivity could be unveiled. *In vitro* knockdown of *Cntnap2* in mouse cortical neurons also causes reduced dendritic morphology, thus suggesting that *Cntnap2* has a role in regulating neurite outgrowth (Anderson et al., 2012). It is also involved in regulating neuroplasticity, specifically the formation and stabilization of dendritic spines (Gdalyahu et al., 2015). We Based on findings in rodent models and in humans bearing the *Cntnap2* risk allele, we hypothesize that reduction of

the transcript in zebra finch song nuclei during the sensorimotor phase of vocal learning will alter dendritic branching as well as long range connectivity. At the behavioral level, the contribution of *Cntnap2* to song learning can be evaluated for each song control nucleus. The sexually dimorphic expression of *Cntnap2* mRNA and protein in the brains of developing zebra finches (Panaitof et al., 2010; Condro and White, 2013) enables us to prioritize testing of these nuclei. Specifically, the primary motor control nucleus for song, the robust nucleus of the arcopallium (RA), provides a compelling target, based on its similar gene expression profile to that of human laryngeal motor cortex (Pfenning et al., 2014). In both songbirds and humans respectively, the RA and laryngeal motor cortical areas make direct contact with the motor neurons involved with learned vocal production, a connection that is lacking in non-human primate and non vocal learning birds. Thus, preventing the sexually dimorphic enhancement of *Cntnap2* expression in developing male zebra finch RA promises to provide clues as to its specific function in human speech.

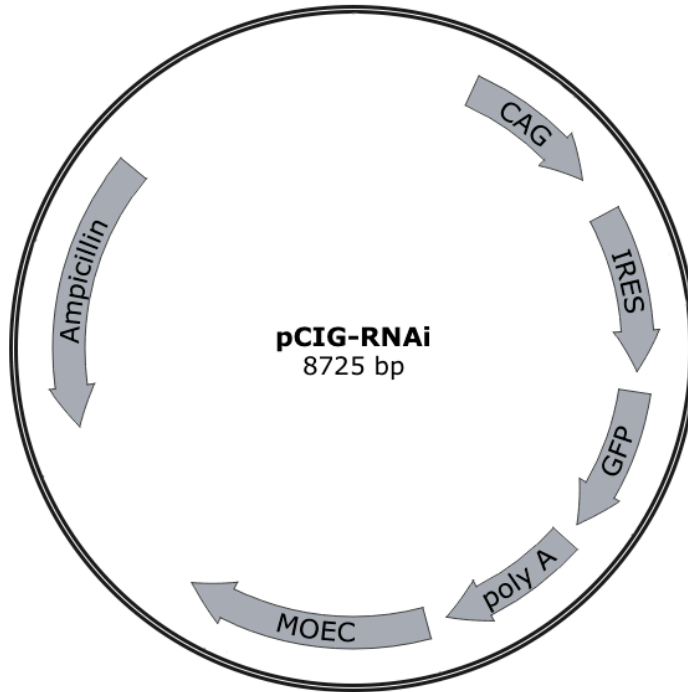
Acknowledgements

We thank Drs. Bennett Novitch and Zachary Gaber for providing the pCIGRNAi plasmid and advice regarding the cloning of shRNA constructs. Thanks to Dr. Julie E. Miller for advice in preparing zebra finch primary telencephalic cell cultures.

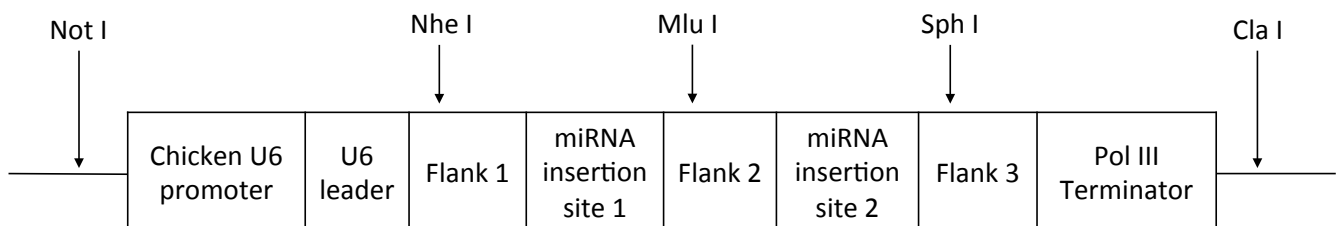
Figures

Figure 2-1: RNA interference vector design.

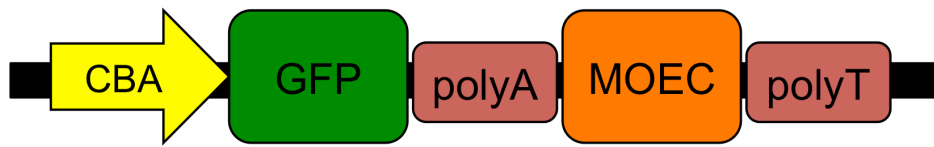
A



B

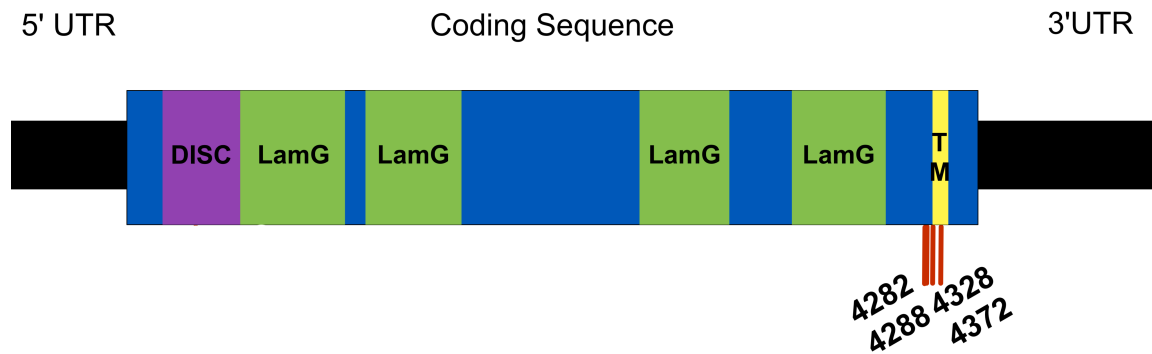


C



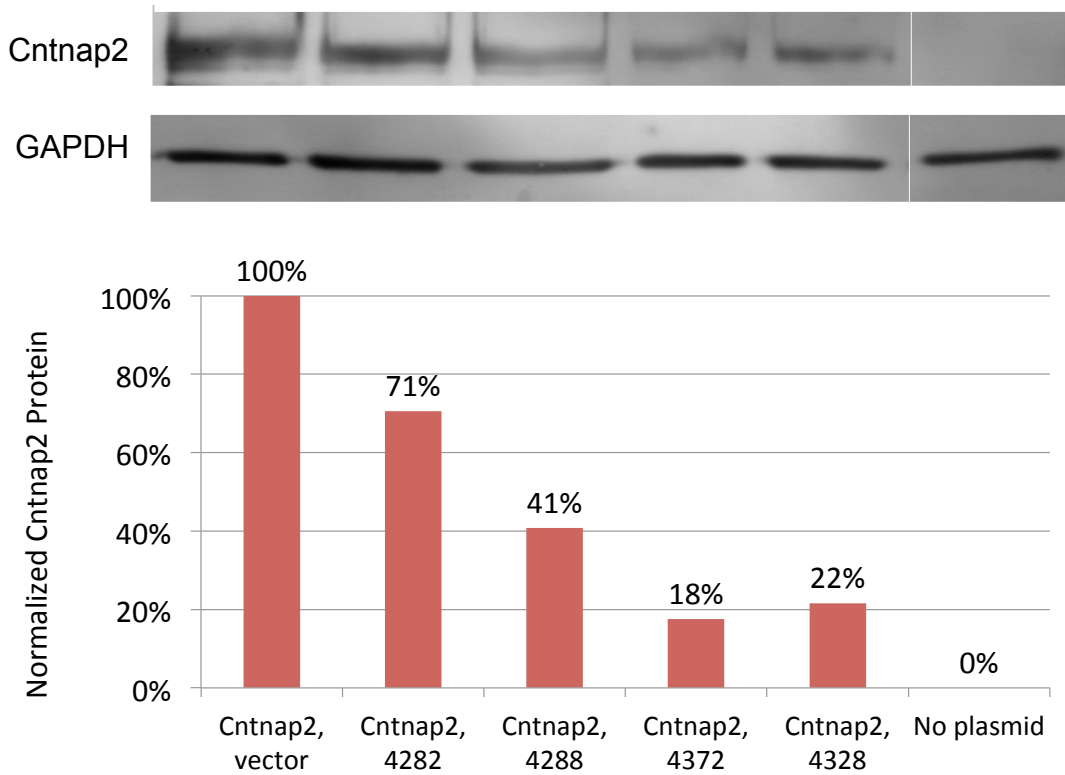
A) Schematic of pCIG-RNAi vector. B) Schematic of MOEC and restriction enzymes sites on MOEC. C) Schematic of “AAV-KD” and “AAV-shGEN”.

Figure 2-2: Schematic of zebra finch *Cntnap2* cDNA (NM_001193337.1).



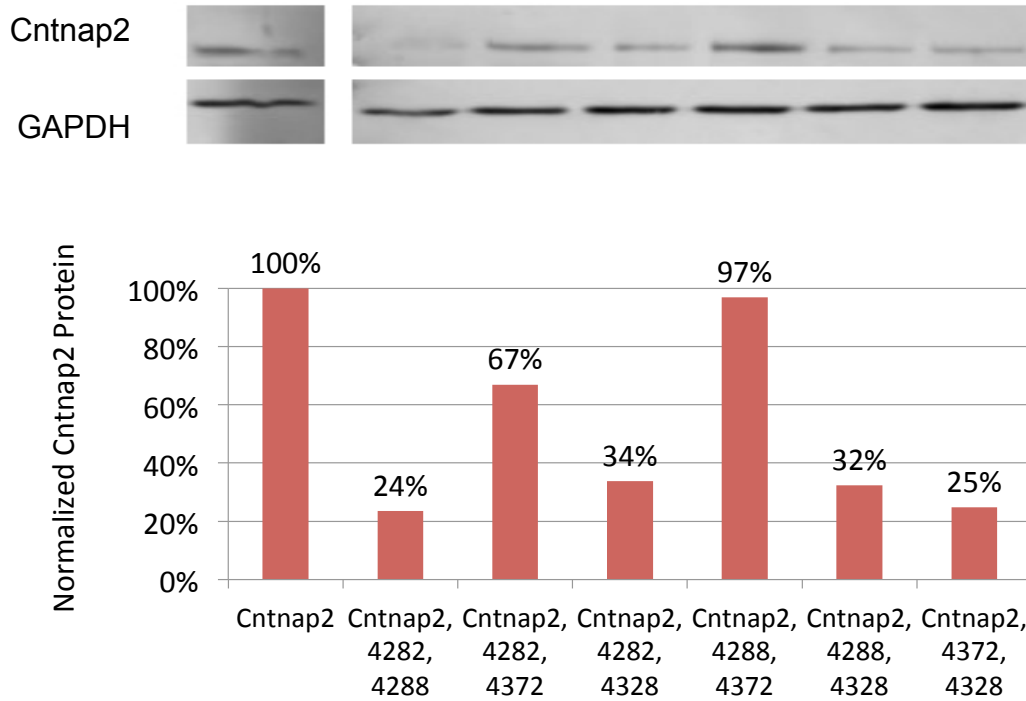
Discoidin (DISC) and laminin G (LamG) protein domains, and the transmembrane (TM) region are superimposed over their corresponding sequences on the cDNA. Target sequences of each shRNA construct in Table 2-1 are indicated by red lines.

Figure 2-3: Single-shRNA constructs effectively reduce zebra finch Cntnap2 in HEK 293 cell culture.



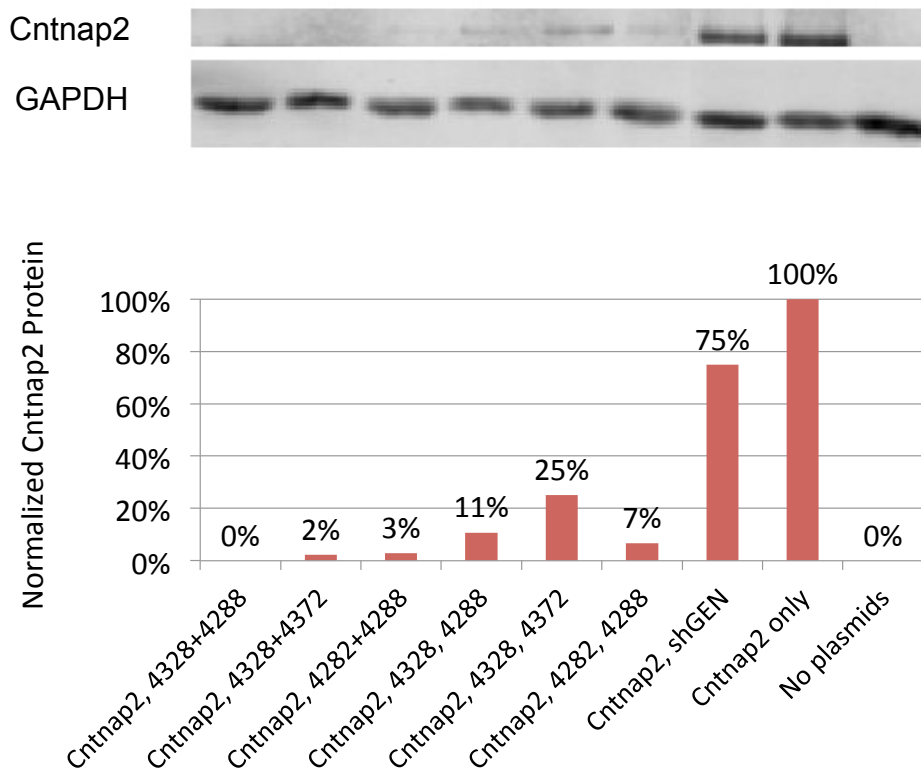
HEK 293 cells co-transfected with individual shRNA constructs and zebra finch Cntnap2 at a 9:1 ratio show that 4328 and 4372 prevent >70% of Cntnap2 expression.

Figure 2-4: Combinations of single-shRNA constructs effectively reduce zebra finch Cntnap2 in HEK 293 cell culture.



Pairs of single-shRNA constructs were co-transfected with Cntnap2 at a 4.5:4.5:1 ratio. Several combinations of shRNAs reduce Cntnap2 expression by >70%.

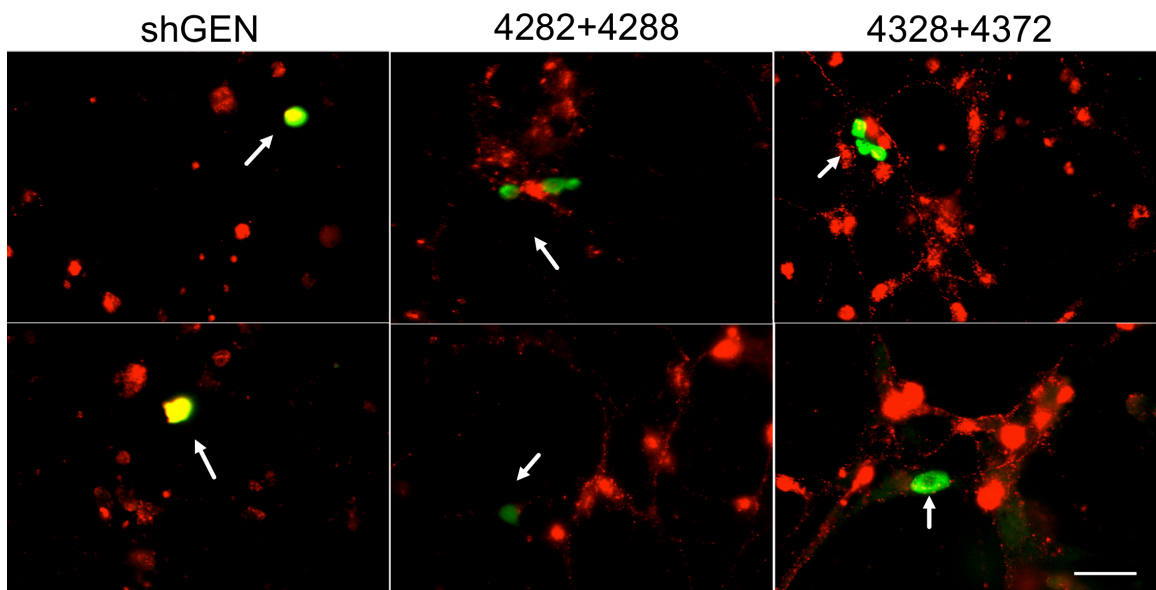
Figure 2-5: Double-shRNA constructs effectively reduce zebra finch Cntnap2 in HEK 293 cell culture.



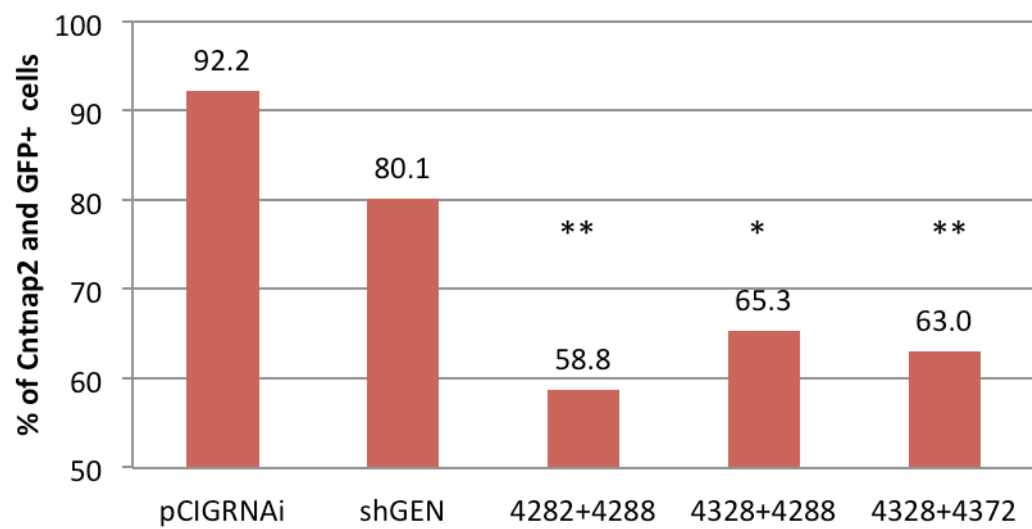
The double-shRNA constructs reduce expression by >95%, a larger effect than that obtained with the combinations of single-shRNA constructs.

Figure 2-6: All three pairs of shRNAs decreased expression of Cntnap2 in zebra finch primary telencephalic cultures.

A



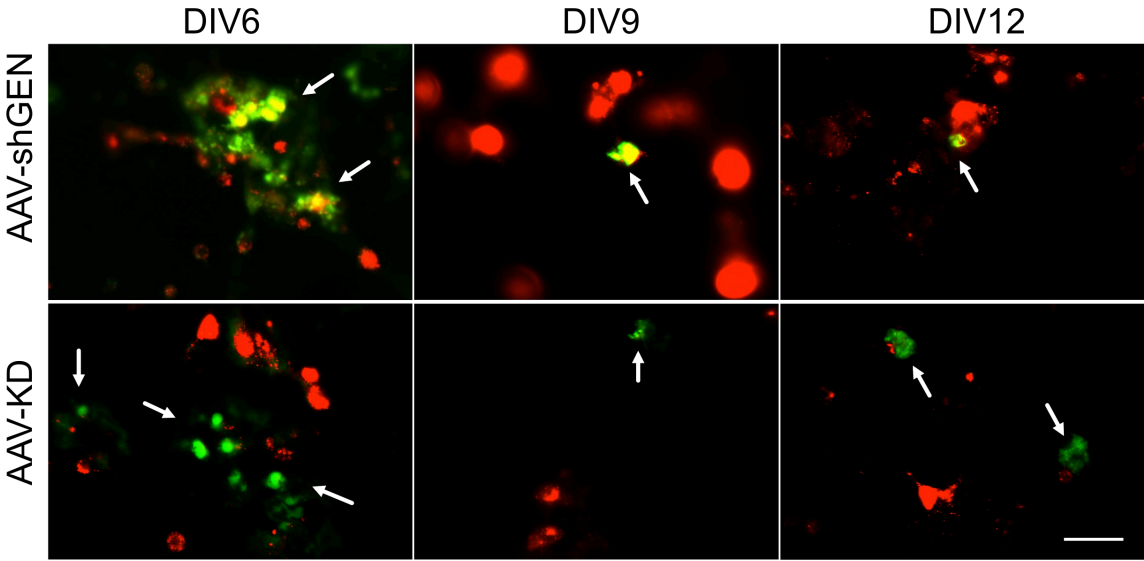
B



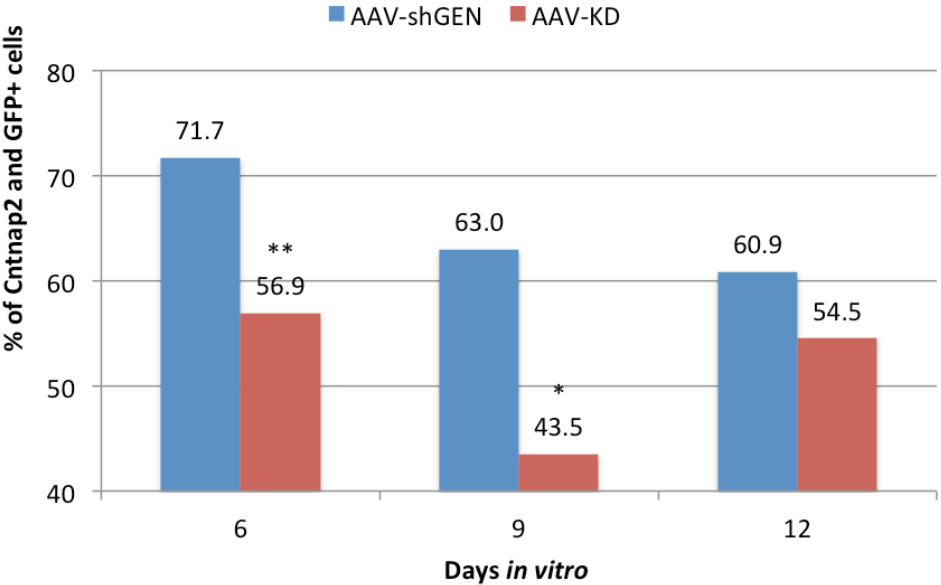
A) Exemplars of immunocytochemical results of zebra finch neuronal primary cultures transfected by different shRNA constructs. Red = Cntnap2, green = GFP. Scale bar: 20um. B) Quantification of Cntnap2 expression in zebra finch neuronal primary cultures. χ^2 test, * $p < 0.005$, ** $p < 0.0001$.

Figure 2-7: AAV-KD reduced Cntnap2 expression in zebra finch neuronal primary telencephalic cultures, relative to AAV-shGEN.

A



B



A) Exemplars of immunocytochemical results of zebra finch primary telencephalic cultures transduced by AAV-KD and AAV-shGEN. Red = Cntnap2, green = GFP. Scale bar: 20um. B) Quantification of Cntnap2 expression in zebra finch neuronal primary cultures. χ^2 test, * $p < 0.01$, ** $p < 0.0001$.

Tables

Table 2-1: shRNA construct names and target sequences designed to knock down zebra finch *Cntnap2*.

shRNA	Target Sequence
4282	CAAGCTATAGGAGATGGAGTTA
4288	ATAGGAGATGGAGTTAACAGAA
4328	GTCATCGCAGTGGTGATCTTCA
4372	TGTTCTGATCCGCTACATGTT
shGEN	CAGTCGCGTTTGCGACTGGATA

Table 2-2: Quantification of Cntnap2 expression in different groups of zebra finch neuronal primary cultures transfected with shRNA constructs.

Plasmids	Cntnap2 & GFP+	GFP+	%Cntnap2 & GFP+
pCIGRNAi	237	257	92.2
shGEN	306	382	80.1
4282+4288	235	400	58.8
4328+4288	49	75	65.3
4328+4372	281	446	63.0

Table 2-3: Quantification of Cntnap2 in different groups of zebra finch neuronal primary cultures transduced with AAV.

Time	AAV-shGEN			AAV-KD		
	Cntnap2 & GFP+	GFP+	%Cntnap2 & GFP+	Cntnap2 & GFP+	GFP+	%Cntnap2 & GFP+
DIV6	294	410	71.7	215	378	56.9
DIV9	182	289	63.0	30	69	43.5
DIV12	28	46	60.9	66	121	54.5

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Chapter 3: Attenuation of Cntnap2 Expression in a Key Vocal Control Nucleus of the Zebra Finch Song System

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Abstract

Mutations in contactin associated protein-like 2 (Cntnap2) are associated with cortical dysplasia-focal epilepsy, autism spectrum disorder, and specific language impairment. Across these diffusely debilitating disorders, deficits in speech and language are a common theme. Songbirds are useful models for the study of human speech disorders due to the significant similarities between the learning of song and speech. The laryngeal motor cortex, a language control region in the human brain shares striking similarities with the robust nucleus of the arcopallium (RA), the primary vocal motor control nucleus in the songbird brain, both in terms of gene expression profiles and in making direct neuronal projections onto the motor neurons that control the muscles of phonation. In the zebra finch (*Taeniopygia guttata*), song learning and its underlying neural circuitry is sexually dimorphic; males, but not females, learn to sing courtship songs and do so using a fully interconnected set of song-dedicated brain regions. Within RA, Cntnap2 expression becomes sexually dimorphic over the course of song learning: males retain high levels whereas expression declines in females. To begin to assess the role of Cntnap2 specifically in vocal learning, here, we stereotaxically injected an adeno-associated virus (AAV) bearing a previously developed shRNA construct into zebra finch RA to attenuate the Cntnap2 expression during the sensorimotor phase of vocal learning. We found that knocking down Cntnap2 in RA caused inaccurate imitation and a high percentage of omission of the tutor song but did not interfere with the bird's ability to modify its song over the course of sensorimotor learning. These results suggest that among Cntnap2's many functions within the nervous system, its expression within the cortical vocal control region alone is critical for accurate vocal imitation.

Introduction

Like other songbirds, zebra finches possess a distinct set of interconnected brain nuclei dedicated to vocal learning and production. This so-called song control circuitry includes the anterior forebrain pathway (AFP), which is important for song learning in juveniles and song maintenance and plasticity in adults, and the posterior descending pathway, which is required for song production throughout life (Brainard & Doupe, 2000; Kao, Doupe, & Brainard, 2005; Scharff & Nottebohm, 1991). Neurons in the HVC (acronym used as a proper name), a premotor vocal control nucleus, directly project to the robust nucleus of the arcopallium (RA) (Nottebohm, 2005; Nottebohm, Stokes, & Leonard, 1976) and indirectly project to the RA through basal ganglia nucleus Area X, the medial nucleus of the dorsolateral thalamus, and the lateral magnocellular nucleus of anterior nidopallium (LMAN) in the AFP. The AFP is homologous to basal ganglia-thalamo-cortical circuit loops in mammals. The unique clustering of song dedicated neurons within the songbird brain provides a remarkable opportunity to test the role of genes implicated in human speech learning, for their function in vocal learning circuitry.

Contactin-associated protein like 2 (Cntnap2, also abbreviated as Caspr2) is broadly implicated in language impairments. For example, a group of Old Order Amish harbor a mutation that results in cortical dysplasia-focal epilepsy, and the affected children exhibit language regression (Strauss et al., 2006). Among the general population certain Cntnap2 alleles are risk variants for autism spectrum disorder (ASD) (Arking et al., 2008), and other alleles correlate with the age at which the first word is spoken (Alarcón et al., 2008). The known role of Cntnap2 is to cluster voltage-gated potassium channels (VGKCs) at the nodes of Ranvier throughout the nervous system (Poliak et al., 1999). Given this general role, it is interesting to evaluate the specific contribution of Cntnap2 to vocal learning and production, as can be tested

using the songbird song control circuitry. To do so, we previously developed an adeno-associated virus bearing shRNAs to knockdown zebra finch *Cntnap2* (AAV-KD). Using this construct, we can specifically knock down *Cntnap2* in individual song control nuclei.

In cortical song control circuitry of adult male zebra finches, *Cntnap2* is enriched in both RA, required for song production, and LMAN, involved in song learning. In contrast to males, adult females do not learn songs and have a moderate level of *Cntnap2* in RA and LMAN. Interestingly, in young females (≤ 35 d) *Cntnap2* is enriched in RA to the same degree as for males, but then declines to the level of the surrounding arcopallium over the course of three weeks. This reduction in gene expression coincides with the onset of sensorimotor period of song learning in males (beginning ~ 30 days post hatch; 30d), a time at which the juvenile male begins to practice singing. The percentage of cells expressing the protein in female RA decreases at this time point (Condro & White, 2013; Panaitof, Abrahams, Dong, Geschwind, & White, 2010).

Songbird vocal learning shares significant similarities to human speech learning, including at the circuitry level. Projection neurons from RA are similar to layer 5 pyramidal neurons in human cortex whose axons descend below the telencephalon to synapse directly onto motor neurons (Jarvis, 2004), and LMAN shares similarities with the mammalian prefrontal cortex (Kojima, Kao, & Doupe, 2013). Recent transcriptomic studies indicate that vocal control regions of the human and zebra finch brain share greater similarity in gene expression profiles than do the corresponding regions of non-human primates, and non-vocal learning birds, respectively. Specifically, the primary motor control nucleus, RA shares a similar gene expression profile to that of human laryngeal motor cortex (Pfenning et al., 2014), whereas the similarity between LMAN and Broca's area was less significant. In both songbirds and humans respectively, the RA and laryngeal motor cortical areas make direct contact with the motor

neurons involved with learned vocal production, a connection that is lacking in non-human primates and non vocal learning birds.

The sexually dimorphic expression of *Cntnap2* mRNA and protein in RA supports the hypothesis that *Cntnap2* expression in RA is important for vocal learning in zebra finch. Considering the great similarities shared by RA and human laryngeal motor cortex, knocking down *Cntnap2* in RA will provide clues to its function in human speech. In order to assess the role of *Cntnap2* to vocal learning, I stereotaxically injected AAV-KD and control constructs specifically into RA at the onset of the sensorimotor phase for vocal learning. Based on findings in rodent models and in humans bearing the *Cntnap2* risk allele, we hypothesize that reduction of the transcript in zebra finch song nuclei during the sensorimotor phase of vocal learning will impair vocal learning. Such impairment could reflect a complete inability to modify song, or a more refined deficit such as the inability to copy the tutor song.

Methods and materials

Subjects

All animal use and experimental procedures were in accordance with NIH guidelines for experiments involving vertebrate animals and approved by the UCLA Institutional Animal Care and Use Committee. Zebra finches (n=17 male, n=17 female) used in this study were obtained from our breeding colony. Sex was determined based on sexually dimorphic plumage. Juvenile (~30d), male zebra finch (“pupils”) were moved to sound attenuation chambers (Acoustic Systems) along with both parents and any clutch mate siblings. At this time, stereotaxic neurosurgeries were performed on all male siblings as described below, after which they were returned to their families. All surgeries were done between 28d to 36d. Vocalizations from the adult male parent (“tutor”) were recorded at this time in the presence of the female parent and the juveniles. At 40d, the time at which juveniles can feed themselves, each pupil was separated from his family and placed within a sound attenuation chamber along with an adult, unrelated female to enable social interactions. Vocalizations were recorded continuously from 40 to 90d. By 90–95d, all pupils were overdosed via isoflurane inhalation, and their brains extracted and prepared for histological analysis.

Surgical procedures

Zebra finches were offered an oral dose of Metacam® (Boehringer Ingelheim Vetmedia, Inc. St. Joseph, MO) for preoperative analgesia and then anesthetized with 2-4% isoflurane carried by oxygen using a Universal Vaporizer (Summit Anesthesia Support, Menlo Park, CA)

for the duration of the surgery. Prior to surgery, a stereotaxic apparatus was prepared by setting a guide pipette to 0.3mm caudal to interaural zero, i.e. between the ear bars that are used to hold the head in place. Following anesthetic induction, the bird was placed on a homeothermic blanket mounted onto the stereotaxic apparatus and bland ophthalmic ointment applied to the eyes. The cranial feathers were removed to expose the scalp, which was then cleaned using povidone-iodine and a surgical drape was applied. In order to preserve vascular flow to the region, a semi-circular incision was made originating and terminating at the caudal edge of the exposed scalp. The scalp flap was then folded back over a Gelitasponge (Gelita Medical, Amsterdam, Netherlands) moistened with sterile saline, to expose the skull. The head angle was adjusted so that the guide pipette was positioned directly above the exposed midsagittal bifurcation ($\sim 18-22^\circ$). $\sim 1\text{mm}^2$ windows were cut in the skull 1mm caudal and 2.4mm lateral from the midsagittal bifurcation. A glass electrode mounted on a Nanoject II (Drummond Scientific, Broomall, PA) was filled with high titer (10^{13} vector genomes) AAV1 (Virovek, Hayward, CA), and lowered into the brain 2.0 mm from the surface of the brain. After a resting period, 20 injections of 27.6 nL each were made 15s apart for $\sim 0.5\mu\text{L}$ total volume per hemisphere. 10 minutes after the last injection, the electrode was withdrawn and the procedure was repeated on the contralateral hemisphere. After each procedure, the scalp was closed and sealed with Vetbond (Fisher Scientific). Each bird was given 2-5 drops of oral analgesic Metacam® after recovery from anesthesia.

Histological analysis

At 90-95d, birds were overdosed with isoflurane, then transcardially perfused with

warmed saline followed by 4% paraformaldehyde in phosphate buffered saline (PBS). Brains were dissected out and cryoprotected in a 20% sucrose solution. 30 μm thick sections were cut in either the coronal or sagittal orientation on a cryostat (Leica Microsystems, Bannockburn, IL) and thaw mounted onto microscope slides (Colorfrost® Plus; Fisher Scientific, Pittsburgh, PA) in a manner that produced replicate sets of adjacent or near-adjacent sections, then stored at -80°C until use. Histological analysis was performed using standard immunohistochemistry.

Sections were warmed to room temperature. A liquid repellent border was drawn along the edges of the slide. Sections were then washed for 15 min with tris-buffered saline (TBS). They were blocked with 10% goat serum in TBS with 0.1% Triton-X for 1hr, then incubated overnight at 4°C with anti-NeuN antibody (anti-NeuN; Millipore, Temecula, CA, Cat. No. MAB377) diluted at 1:1,000 and anti-GFP antibody (anti-GFP; Millipore, Temecula, CA, Cat. No. AB10145) diluted at 1:500 in TBS with 1% goat serum. Unbound primary antibody was washed away with TBS, then sections were incubated in goat-anti-mouse IgG Alexa Fluor® 555 secondary antibody (Life Technologies, Grand Island, NY, Cat. No. A21422) diluted at 1:1,000 and goat-anti-rabbit IgG Alexa Fluor® 488 secondary antibody (Life Technologies, Cat. No. A11008) diluted at 1:1,000 in TBS with 1% goat serum for 2hr. Slides were mounted with glass coverslips using Prolong Antifade Gold Reagent (Life Technologies, Cat. No. P36934) and stored at 4°C until use.

Image acquisition and analysis

Images were acquired using an Axio Imager.A1, with an AxioCam HRm digital camera (Carl Zeiss Inc., Oberkochen, GE). Axiovision software (Carl Zeiss Inc.) was used to optimize

photomicrographs to remove background, improve brightness and contrast, and to pseudocolor the images. NeuN is represented here in red; GFP is represented in green. Adjustments were made to the entire image and not to selective subregions.

Pictures of brain sections from AAV treated birds were taken. One section from each hemisphere of each bird with the relative most GFP+ cells within RA was chosen. The border of RA was determined based on the density of NeuN immunoreactivity, The transduction rate was determined by counting the total number of GFP+ and NeuN+ cells within RA and then dividing by total number of NeuN+ cells within the selected RA section.

Song recording

Sounds were recorded using either a Countryman EMW omnidirectional lavalier microphone (Countryman Associates) or a Shure SM58 microphone and digitized using a PreSonus Firepod (44.1 kHz sampling rate, 24 bit depth). Recordings were acquired and song features quantified using Sound Analysis Pro (SAP) 2011 software (Tchernichovski, Nottebohm, Ho, Pesaran, Mitra, 2000). Although the investigator knew the group allocation during the experiment, this automated software was used to derive all measures of song learning and acoustic features, avoiding subjective assessment.

Song analysis

Song analysis generally followed that used by Heston & White (2015), as follows: Songs were manually hand-segmented into motifs and individual syllables by the experimenter, and

then analyzed in a semi-automated manner using Sound Analysis Pro (SAP; cite Tchernichovski). Motifs were identified as repeated units of song composed of multiple syllables. Introductory notes were excluded. Canonical and noncanonical renditions of motifs were included in the analysis to capture the full range of singing behavior. A syllable was identified as a sound element that is separated from other syllables by local minima in the amplitude (Immelmann, 1969).

Motif analysis to tutor. We used SAP to quantify how well pupils imitated their tutor's motif using similarity scores obtained from 200 asymmetric pair-wise comparisons of 20 renditions of the pupil's typical motif with 20 renditions of the tutor's motif. The same 20 renditions of the tutor's motif were used for all pupils of the same tutor. Asymmetric comparisons analyze the spectro-temporal similarity of syllables without respect to their position within a motif. This operation is well suited to the analysis of motifs because it measures large timescale resolution of acoustic similarity and makes no assumptions about syllable order. We report the upper-third quartile score from these comparisons so as not to underestimate the percentage of tutor song copied. Automated analysis was supplemented by manual counting of imitated, omitted, and improvised syllables. The numbers were normalized to the total number of syllables in the tutor song to obtain the percentage of copied song and the percentage of improvised song.

Analysis of song development. To determine the developmental trajectory of vocal imitation, songs recorded on 55, 60, 65, 70, 75, 80, 85 and 90 d (± 2 d; in several cases recordings were unavailable due to technical issues) were analyzed. Twenty motifs were compared asymmetrically to a single tutor motif (this motif was chosen to be representative of the tutor's vocal repertoire and the same motif was used for comparison with each of that tutor's pupils).

The same group of songs was also compared asymmetrically to 20 renditions of motif from 90 d of its own song. The upper-third quartile score from these comparisons is reported.

Syllable analysis to tutor. The similarity and accuracy of individual syllables of the pupil's song to the tutor's syllables were quantified using symmetric comparisons. Symmetric comparisons analyze the spectro-temporal similarity and accuracy from the beginning to the end of the two syllables under investigation. This operation is well suited for the analysis of syllables for which the sound elements have already been isolated and can be assumed to begin and end at corresponding time points. 20 renditions of a tutor syllable were compared with 20 renditions of the corresponding pupil syllable, generating 400 unique comparisons. Each copying metric was represented by the median of these 400 comparisons.

Statistics

The bootstrap one-way ANOVA was performed throughout our analysis to determine if there is any difference between each group. F-statistics were generated for the actual data set and then compared with a distribution of 10,000 F-statistics calculated by resampling the original data under assumption of the null hypothesis to determine whether the "AAV-KD" treated group has a behavioral effect compare to the "AAV-shGEN" treated group and the non-injected group.

Results

Stereotaxic targeting of AAV constructs

AAV was injected into the brains of juvenile male zebra finches targeting RA bilaterally. The treated birds were then sacrificed after 90d to evaluate the result of surgery. Figure 3-1 shows an exemplar of a successful targeting with AAV-shGEN; GFP signal represents the AAV transduced cells while NeuN signal is a neuron specific marker. The border of nuclei was determined based on the density of NeuN immunoreactivity, which is more pronounced within the nucleus. In this representative exemplar, we observed a large number of GFP labeled cells in RA, with many long labeled processes. The co-localization of GFP with NeuN signal shows the AAV successfully transfected neurons in RA (Figure 3-1B, blue panel). The average transduction rate of neurons in both RAs of this bird is 30%. GFP labeled axon tracts extending from RA into the hindbrain indicates the AAV successfully transfected RA projection neurons (Figure 3-1B, orange panel). GFP signal is also found in the dorsomedial nucleus of the intercollicular complex (DM) in midbrain. The lack of co-localization of NeuN with GFP signals in the DM also indicates that neurons in the DM are not being transfected, and that the GFP signal is due to projection neurons from the RA innervating the DM, which is a known target of RA {Vicario:1991db, Figure 3-1B} purple panel). No GFP signal was detected in other relevant song nuclei including HVC or LMAN suggesting that the transduction was largely restricted within RA, and that the AAV is not being retrogradely transported to other regions.

Histological analysis

We have confirmed that we can target RA in juvenile zebra finches with the designed AAVs and that the expression of AAVs can be detected up to at least two months following injection. Next, we injected AAVs into male siblings to detect the behavioral effect of attenuation Cntnap2 expression in RA. After pupils reached 90d, they were sacrificed and their brains were examined for successful stereotaxic targeting of both RAs. Exemplars of brain sections from three pupils of Tutor A are shown in Figure 3-2. The transduction rate of AAV within each RA in each pupil is A1: 25%, 21%, A2: 8%, 1% and A3: 20%, 21% respectively. No detectable GFP signal was seen in other song nuclei of all pupils. For all the AAV injected birds used in the study, stereotaxic injections were verified through the same process. If the transduction rate in both RA is lower than 20%, that bird was excluded from the behavior analysis. For example, pupil A2 was excluded and considered as a ‘miss’, likely accounting for the higher degree of similarity of this pupil’s song to its tutor (see the section below).

Pupils from four tutors met the criteria described above and thus were involved in the behavior analysis. Tutor A had three pupils. For each trio, two were injected with AAV-KD bilaterally and one was injected with AAV-shGEN bilaterally. Tutor B, C and tutor D each had two pupils. For each pair, one was injected with AAV-KD and the other with AAV-shGEN bilaterally. Among all the pupils, pupil A2 was excluded from the behavior analysis because the unsuccessful targeting of RA.

Evaluation of song learning

Exemplars of the song motifs from four tutors and their pupils’ 90d songs are shown in Figure 3-3. Pupils that were successfully injected with AAV-KD all present shorter motifs with

many omitted syllables compared to their tutor's song. They exhibit a lower motif similarity score to the tutor song. Pupils that were successfully injected with AAV-shGEN copied a relatively complete version of their tutor's songs and exhibited a higher motif similarity score. Pupil A2, however, which was injected with AAV-KD but considered as a "miss", copied the tutor song quite well and exhibited a high motif similarity score.

Characterization of song deficits

We first analyzed the 90d 'adult' song of each pupil. The motif similarity score was calculated by SAP semi-automatically to provide an unbiased measure of how well the pupils copied their tutors' songs. We found that the AAV-KD group exhibited a significantly lower similarity score compared to the AAV-shGEN group (Figure 3-4, $p=0.0364$, unpaired one-tailed bootstrap). As an additional control, we also recorded 6 non-injected pupils throughout the sensorimotor learning period. These six birds were raised by another three different tutors. There is also a significant difference between the non-injected group and the AAV-KD group ($p=0.0024$, unpaired one-tailed bootstrap). There are no significant difference between non-injected group and AAV-shGEN group, which indicates the AAV-shGEN and the surgical procedure itself do not change the motif similarity of pupil song. The AAV-KD treated birds exhibit a profound decrease of motif similarity compared to the two control groups.

To further investigate the nature of the learning deficit, we manually examined the motif similarity by counting the number of omitted and improvised syllables in each pupil's song compared to his tutor's song. The similar syllables were manually identified according to the spectrograms. (In the future, this process will be re-tested using a semi-automated method,

namely the Vocal Inventory Clustering Engine; Burkett, Day, Peñagarikano, Geschwind, & White, 2015). The percentage of the tutor's motif that was copied was assessed as the number of copied syllables in pupil's song divided by the total number of syllables in tutor's motif. The percentage of improvised motif equals the number of improvised syllables in pupil's song divided by the total number of syllables in tutor motif. We found that the AAV-KD group copied a significantly smaller part of the tutor motif compared to the AAV-shGEN group ($p=0.0346$, unpaired one-tailed bootstrap) and the non-injected group ($p=0.0274$, unpaired one-tailed bootstrap, Figure 3-5A). All groups exhibited a similarly low percentage of improvisation and no significant difference was found between groups (Figure 3-5B).

For those syllables that were copied from the tutor, we examined the quality of those copies using SAP. Scores of acoustic features were obtained from comparing the pupil syllables to the same syllable in the tutor song and were collapsed within the same bird. The syllable identity score of the AAV-KD group is significantly lower compared to the AAV-shGEN control group ($p=0.0489$, unpaired one-tailed bootstrap) and non-injected group ($p=0.0066$, unpaired one-tailed bootstrap). The syllable similarity score of AAV-KD group is significantly lower compared to AAV-shGEN group ($p=0.0109$, unpaired one-tailed bootstrap, Figure 3-6).

Song development

To analyze the quality of learning during the sensorimotor phase of song development, the motif similarity of the pupil song to the tutor song was examined every five days starting from 55d to create a 'learning curve to tutor'. The learning curve to tutor of 6 non-injected birds shows that all birds exhibit a high similarity score at the end of sensorimotor learning. However,

the speed of achieving this goal can be quite different (Figure 3-7A). Similarly, the virus-injected birds exhibit quite variable learning curves to tutor (Figure 3-7B). When we take look at the birds who shared the same tutor, the AAV-shGEN treated pupil gradually sang more like tutor compared to its AAV-KD treated siblings. We then calculated the mean score of each group and found that the AAV-KD group has a significantly lower motif similarity score starting from 70d compared to the two control groups. The pupil songs on those days were also compared to the bird's own adult song; i.e. song recorded at 90d. We found that the two virus-treated birds followed similar trajectories in attaining the adult version of their songs (Figure 3-8).

Thus, knocking down *Cntnap2* in RA during sensorimotor phase of vocal learning led to inaccurate imitation and a high percentage of omission of the tutor song but did not interfere with the bird's ability to modify its own song over the course of sensorimotor learning.

Discussion

In this study, we investigated the impact of the autism susceptibility gene, *Cntnap2*, specifically on socially-learned vocal communication using the songbird model of vocal learning. We found that attenuation of *Cntnap2* levels in the primary motor control region of the song circuit, RA, disrupted the ability of young males to copy their tutor's songs, but did not impair their ability to modify their songs. We conclude that among *Cntnap2*'s many functions in the nervous system, its presence in the projection neurons that innervate the motor neurons controlling phonation is required for vocal mimicry. Below, we consider some challenges to this interpretation and set the stage for future investigation.

The AAV we developed is not designed to specifically target neurons. Thus, among all the GFP+ cells, there could be other cell types transduced. However, *Cntnap2* is only expressed in neurons (Poliak et al., 1999) so there should not be a functional impact from any non-neuronal cells that received the knockdown construct. The onset of sexually dimorphic *Cntnap2* expression observed in RA at 50d roughly coincides with that for sensorimotor learning which is at 35d in zebra finches (Condro & White, 2013). The AAV requires 1-2 weeks for initial expression, with a peak at 3-4 weeks (Doherty, Schaack, & Sladek, 2011; Papale, Cerovic, & Brambilla, 2009). Ideally, injection of the virus at 15d would begin to knock down *Cntnap2* around the onset of sensorimotor learning. However, birds before the age of 25d are challenging to operate on. Instead, we chose to inject the virus after 25d, which none-the-less resulted in a detectable behavioral effect.

The impaired ability of copying an accurate and complete version of tutor song after knocking down *Cntnap2* in RA is reminiscent of the language regression found in patients

carrying a loss-of-function mutation in CNTNAP2 (Strauss et al., 2006). Presumably these patients retained some functional CNTNAP2 protein. Due to experimental errors in the present study, the transduction rate can be quite variable. Here, birds with a transduction rate greater than 20% in either RA were included in the behavior analysis. However, there may be an intermediate effect in the birds with a transduction rate lower than 20%. The correlation of transduction rate and the song similarity of pupil to tutor remain to be examined. Autistic children harboring *CNTNAP2* risk variants exhibit a delayed age at first word (Alarcón et al., 2008). Thus, in zebra finches, the onset of crystallization can be examined in both virus treated groups to see if knocking down *Cntnap2* in RA of zebra finch creates a similar effect.

The impairment of vocal learning in *Cntnap2* knock down group may due to many reasons. One hypothesis is that knocking down *Cntnap2* in RA impairs learning process by disrupting synaptic dynamics. In juvenile zebra finch, hearing a tutor song could lead to a rapid stabilization, accumulation and enlargement of dendritic spines in HVC. This suggests that rapid spine turn over is associated with the learning capacity at the onset of behavioral learning (Roberts, Tschida, Klein, & Mooney, 2010). *In vitro* knockdown of *Cntnap2* in mouse cortical neurons causes reduced dendritic morphology, thus suggesting that *Cntnap2* has a role in regulating neurite outgrowth (Anderson et al., 2012). It has also been shown in *CNTNAP2*^{-/-} mouse, *CNTNAP2* is involved in stabilizing new dendritic spines in mouse cortex (Gdalyahu et al., 2015). Thus, in addition to the behavioral effect observed here, morphological correlates can also be examined in the future to see whether knocking down *Cntnap2* in RA disrupts the synaptic dynamics.

The other hypothesis is that knocking down *Cntnap2* in RA impairs the learning process by simply by affecting action potential propagation within the motor pathway for song

production. Decreased *Cntnap2* could alter the localization of VGKC's along the axons of RA projection neurons, possibly leading to abnormal stimulation of the downstream 12th tracheosyringeal nucleus (nXIIts) motoneurons and disrupted syringeal control. To determine whether this is the mechanism whereby reduced *Cntnap2* in RA impairs song mimicry in young birds, we could knockdown *Cntnap2* in RA of adult male zebra finch. If this manipulation disrupted the crystallized song of adult birds who are no longer learning, it would suggest a purely motor disruption in young birds as well, rather than a learning deficit per se. It is also possible that *Cntnap2* is required for the maintenance of synaptic circuitry involved in song production, in which case disruption of *Cntnap2* in adulthood will result in deterioration of their song. To disambiguate between synaptic and axonal effects, it may be possible to record from the tracheosyringeal nerve of adult birds who received either AAV-KD or AAV-shGEN.

Another possible study is to examine the unlearned calls of these virus-treated birds. RA contains two functional subdivisions; one set of projection neurons synapse onto midbrain DM and the other directly controls syringeal motoneurons by synapsing onto nXIIts (Vicario, 1991). DM is one of the nuclei of the vocal control system and is known as the midbrain vocal center. It is able to influence the key motor and premotor nuclei involved in patterned respiratory-vocal activity (Wild, Li, & Eagleton, 1997). Although the DM is not required for vocal learning, it is involved in call production of songbirds. The neural information from the RA to the DM can change the distance call pattern of male songbirds (Fukushima & Aoki, 2000; Vicario & Simpson, 1995). Knocking down *Cntnap2* in RA may have changed the neuronal connection between RA and DM and results in a change of call patterns. This possible effect could only be due to changes in motor pathway because calls are not learned vocalizations.

Outside of RA, *Cntnap2* is also highly expressed in another song nuclei, LMAN compared to the surrounding nidopallium. We can use AAV-KD construct to knockdown *Cntnap2* in LMAN to determine the possible behavioral effects. Projection neurons within LMAN send axons to both RA and Area X. Disruption of the circuitry may affect both song development and song production.

Development of a transgenic zebra finch in which *Cntnap2* is knocked down globally is in progress (http://carlosloislab.blogspot.com.es/2014/09/research_27.html, Figure 6). It will be interesting to observe the behaviors of these birds compared with those in our study. We hypothesize that, in addition to possible seizure activity as observed in the affected old order Amish children, these transgenic zebra finches will exhibit a compromised sociability such as fewer interactions with their tutors and/or fewer songs directed to females. They may also show impairment in vocal mimicry. The global knock down obviously disrupts even more aspects of neural function than our targeted approach. By knocking down *Cntnap2* in individual song nuclei of zebra finch, our approach can unravel the specific role of *Cntnap2* on vocal learning and production.

Development of a songbird model to investigate *Cntnap2* opens the door to testing therapeutic interventions that can remediate speech deficits associated with *Cntnap2*-related developmental disorders in humans.

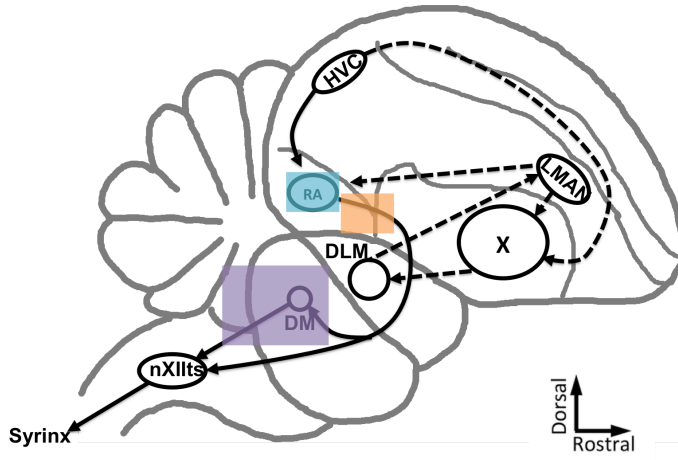
Acknowledgements

Thanks to Jonathan Heston for his advice in handling virus and behavioral analysis and Zachary Burkett for advice in statistics.

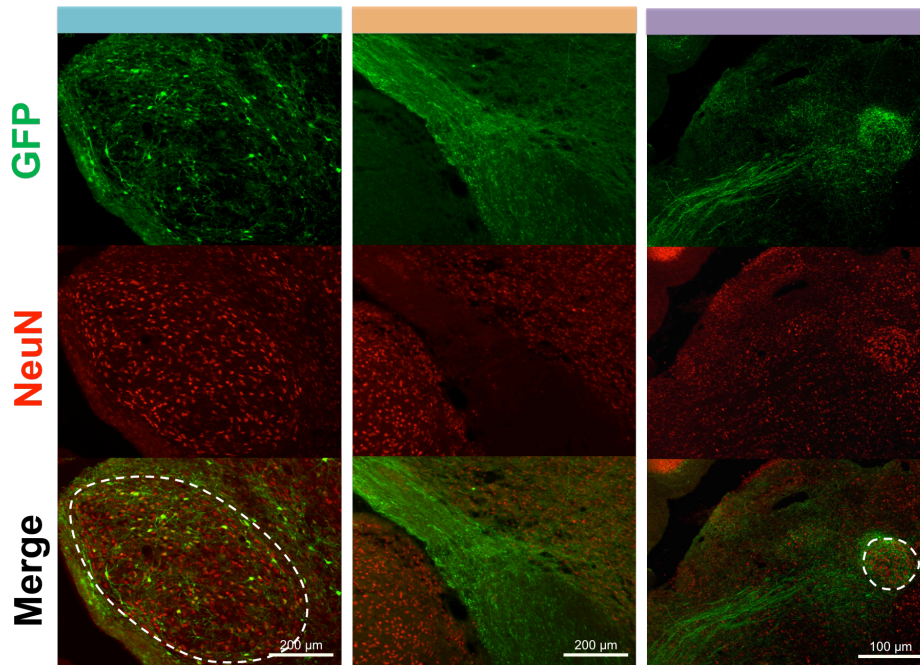
Figures

Figure 3-1: Successful transfection of projection neurons in the RA.

A

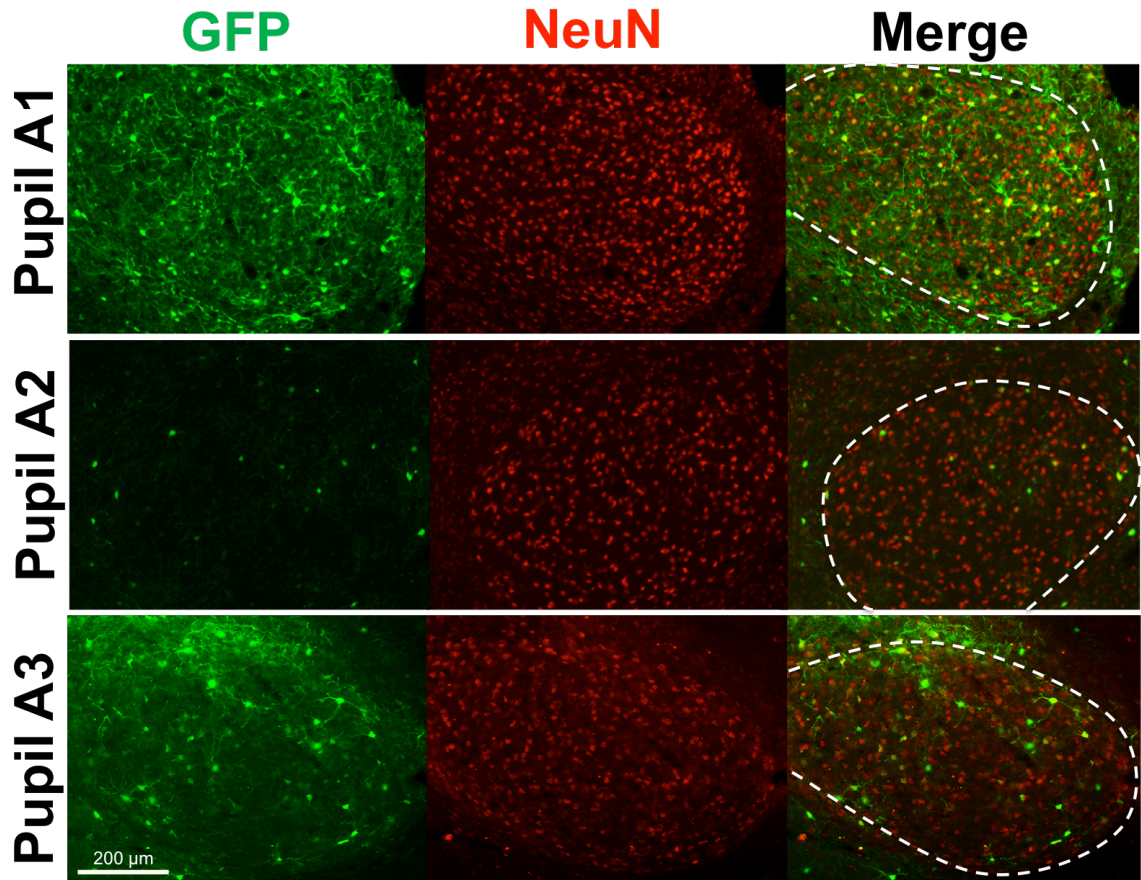


B



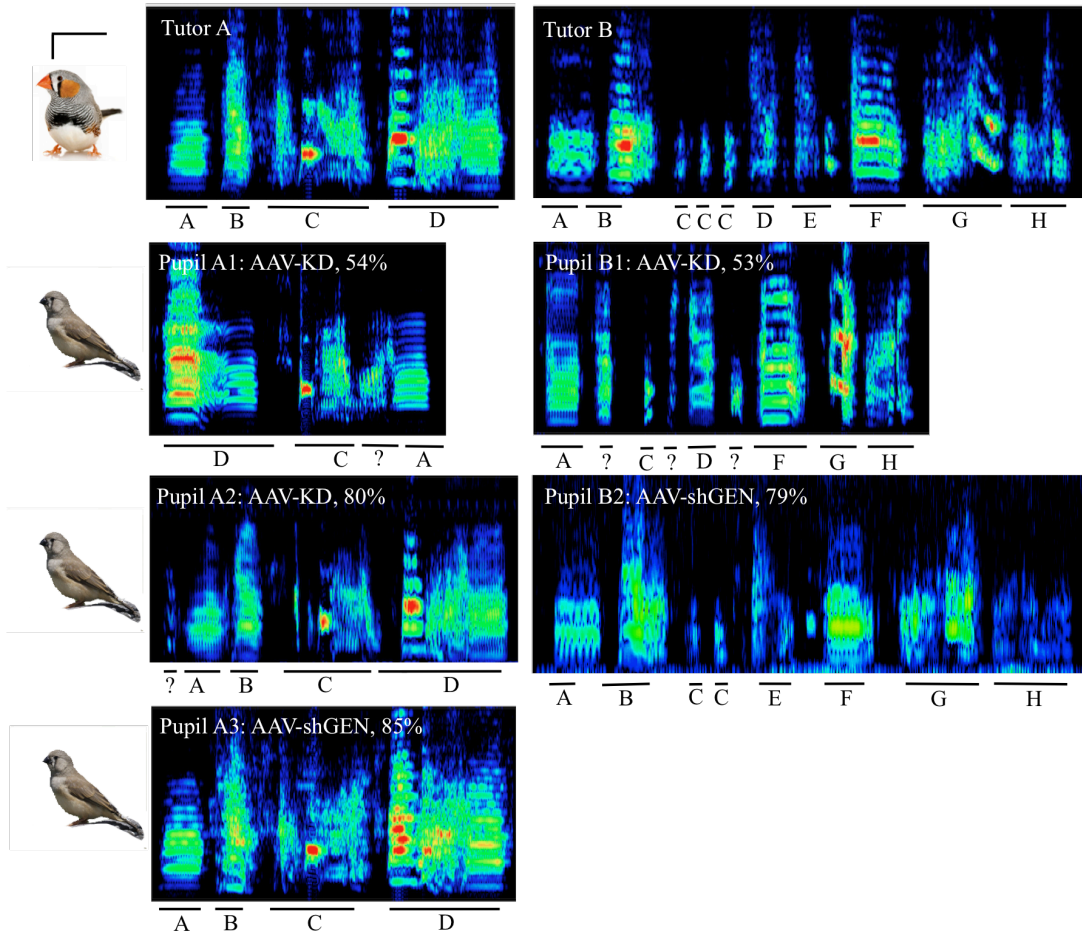
A) Schematic of the song circuit in sagittal section of zebra finch brain. Blue, orange and purple color boxes indicate the areas of interest in B) respectively. B) Representative images of the sagittal sections of zebra finch brain injected with AAV-shGEN. The blue, orange and purple bars correspond to the region of interest indicated in A). Dashed line indicates boundary of the RA and DM. Green: GFP, Red: NeuN.

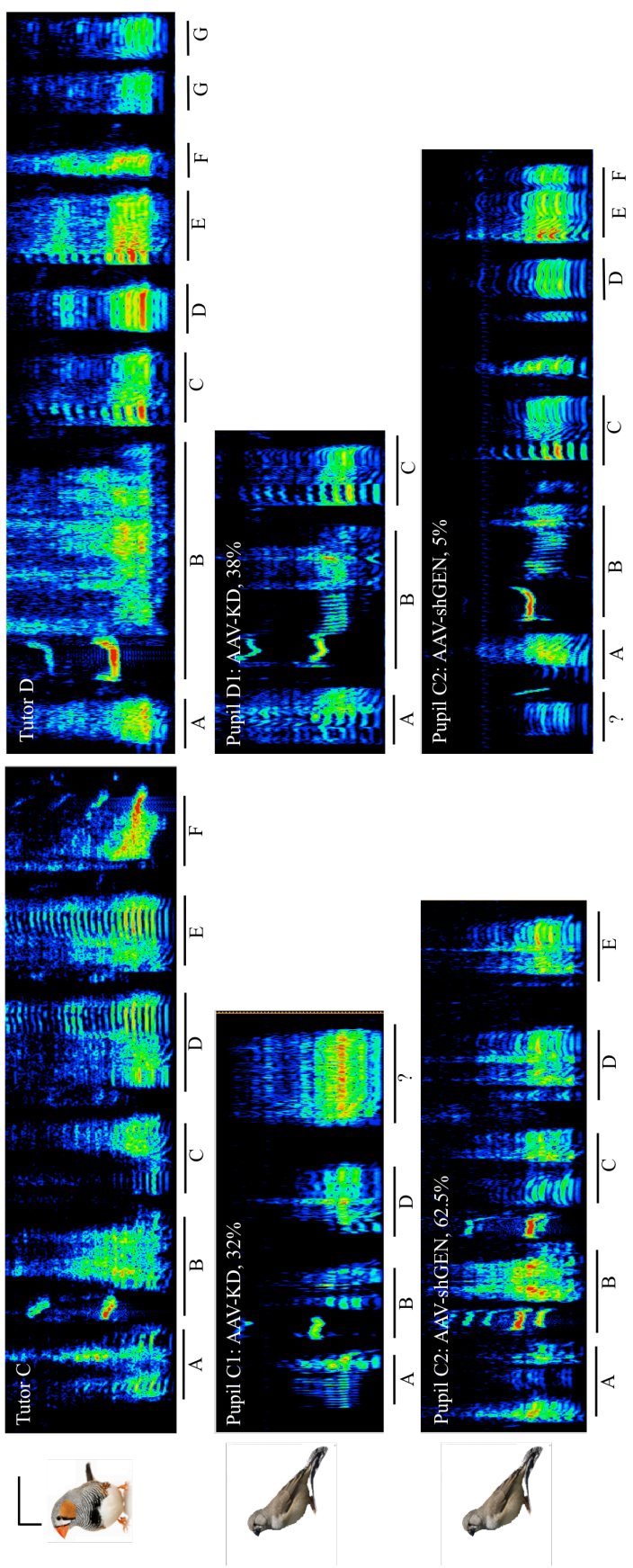
Figure 3-2: Localization of the stereotaxic injections of AAV in three pupils from a given tutor.



Representative images of the sections of three pupils injected with AAV-shGEN or AAV-KD. RA of Pupils A1 and A3 is nicely targeted by AAV whereas RA of pupil A2 is a missed target. Dashed line in the merged image indicates the boundary of the RA. Green: GFP, Red: NeuN.

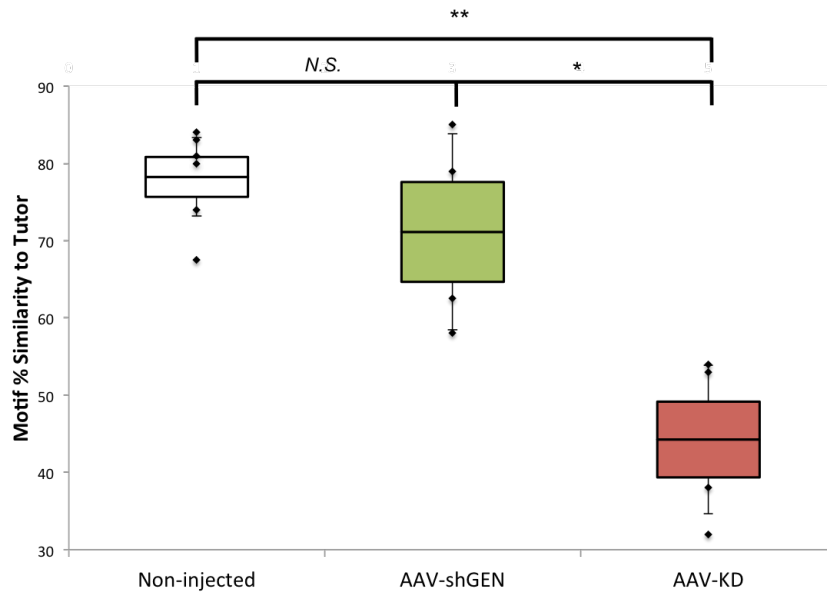
Figure 3-3: Exemplars of the song motifs from four tutors and their pupils, which received stereotaxic injections of AAV.





Tutor A shown here had three pupils that received the stereotaxic injections of AAV. Tutor B, C and D had two. The typical motifs from pupils' 90d songs are showed. The type of virus and the motif similarity to tutor song is marked on each pupil's spectrogram. Syllables that correspond between tutor and pupil motifs are underlined with black bars and identified by letters (question marks indicate unidentifiable syllables). Of note, histological evidence indicates that the AAV-KD injection in pupil A2 mis-targeted RA (see Figure 3-2). Y-axis frequency range = 0 to 16 kHz. X-axis scale bars, 100ms; Y-axis scale bar, 2kHz.

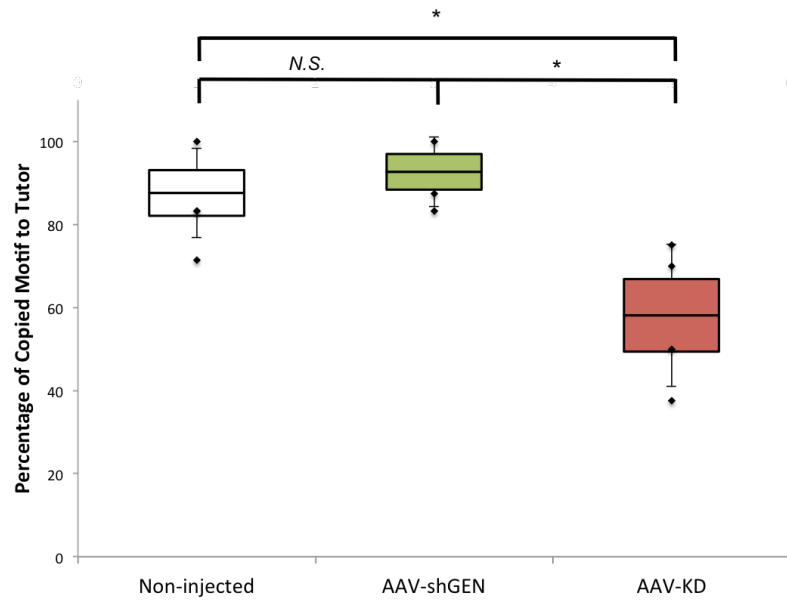
Figure 3-4: Quantification of the motif similarity of each pupil's 90d song to its tutor.



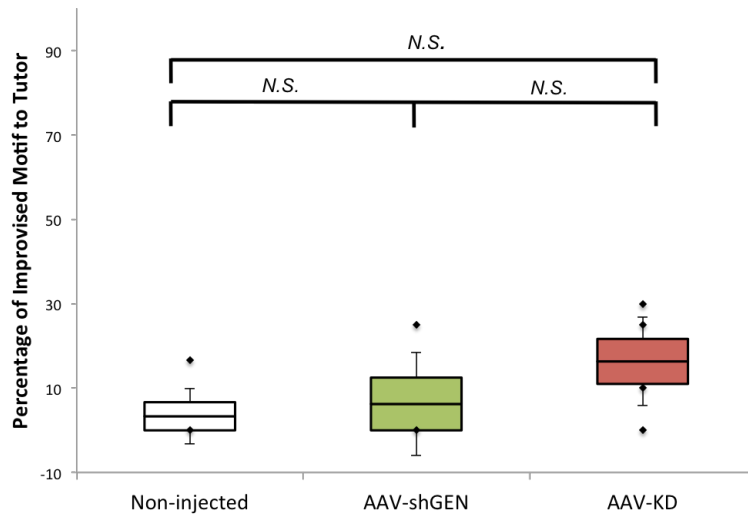
AAV-KD treated birds (red bar) have lower scores than those of AAV-shGEN treated birds (green bars; $p = 0.0364$, unpaired one-tailed bootstrap) and the non-injected group of birds (white bars; $p = 0.0024$, unpaired one-tailed bootstrap). Non-injected, $n = 6$; AAV-shGEN, $n = 4$; AAV-KD, $n = 4$. Midline represents mean, upper and lower bounds of the box represent SE, upper and lower whiskers represent 95% confidence intervals, and points represent individual birds. Two asterisks: $p < 0.005$; one asterisk: $p < 0.05$.

Figure 3-5: Manual analysis of pupil's 90d song to its tutor.

A

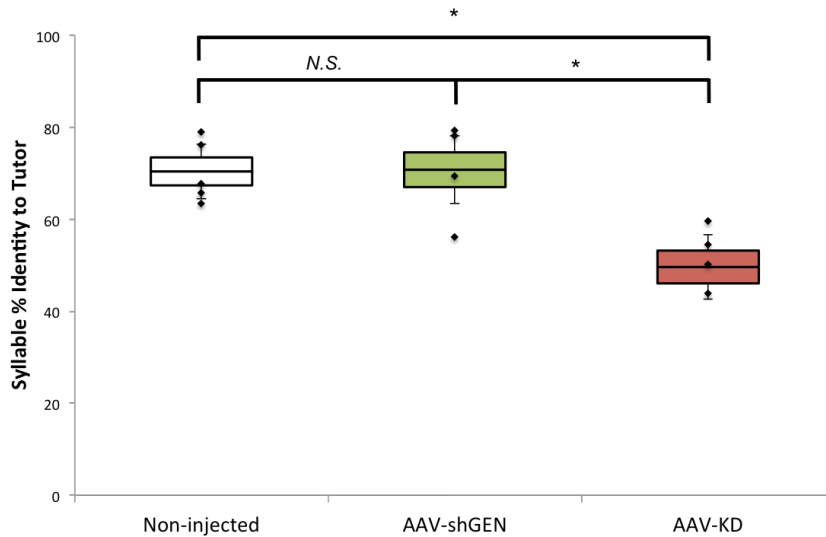


B



A) AAV-KD treated birds (red bar) copied a lower percentage of their tutor's motif compared with both the AAV-shGEN treated birds (green bar; $p=0.0346$, unpaired one-tailed bootstrap) and the non-injected birds (white bar; $p=0.0274$, unpaired one-tailed bootstrap). Non-injected, $n=6$; AAV-shGEN, $n=4$; AAV=KD, $n=4$. Midline represents mean, upper and lower bounds of the box represent SE, upper and lower whiskers represent 95% confidence intervals, and points represent individual birds. B) AAV-KD treated birds (red bar), AAV-shGEN treated birds (green bar) and the non-injected group of birds (white bar) do not exhibit any significant difference in the percentage of improvised song compare to the tutor song ($p>0.05$, unpaired one-tailed bootstrap). Non-injected, $n=6$; AAV-shGEN, $n=4$; AAV=KD, $n=4$. Midline represents mean, upper and lower bounds of the box represent SE, upper and lower whiskers represent 95% confidence intervals, and points represent individual birds.

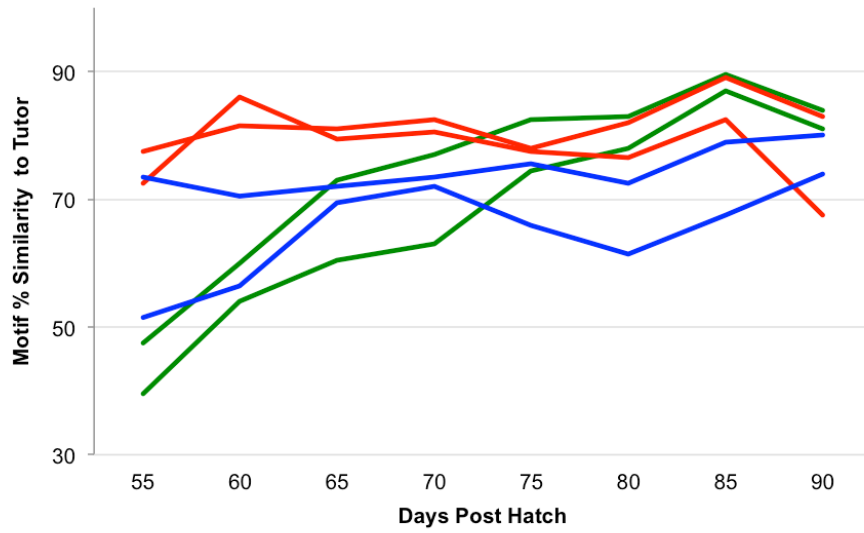
Figure 3-6: Syllable identity of pupil compared to tutor song.



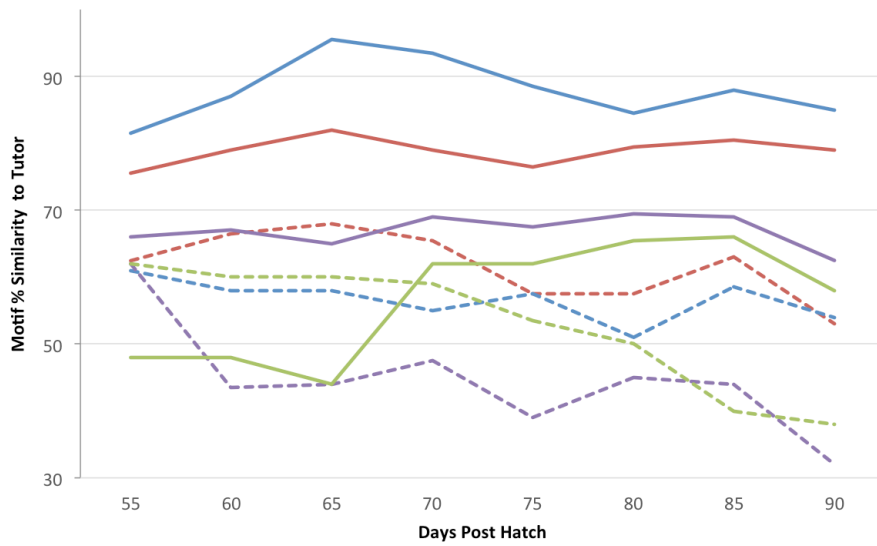
The songs of AAV-KD treated birds (red bar) exhibit lower syllable identity to the tutor syllables compared with both AAV-shGEN treated birds (green bar; $p=0.0489$, unpaired one-tailed bootstrap) and the non-injected group of birds (white bar; $p=0.0109$, unpaired one-tailed bootstrap). Non-injected, $n=6$; AAV-shGEN, $n=4$; AAV=KD, $n=4$. Midline represents mean, upper and lower bounds of the box represent SE, upper and lower whiskers represent 95% confidence intervals, and points represent individual birds.

Figure 3-7: Quantification of motif similarity of pupil song to tutor song.

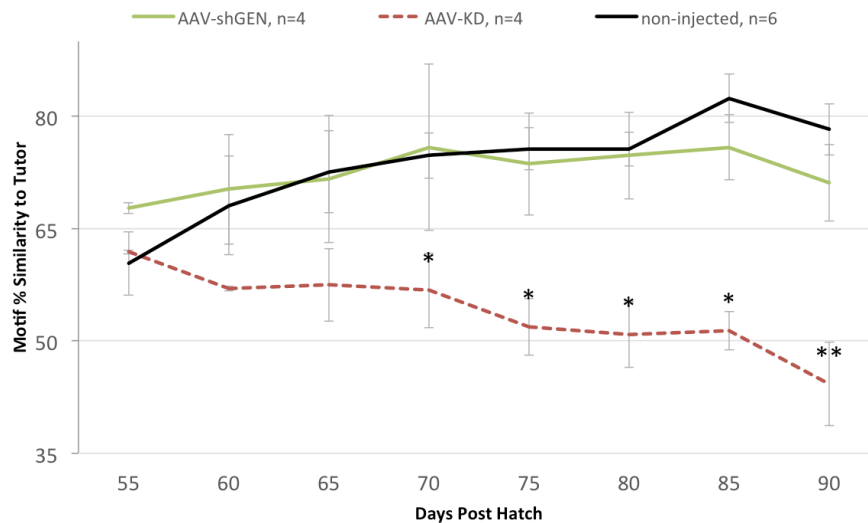
A



B

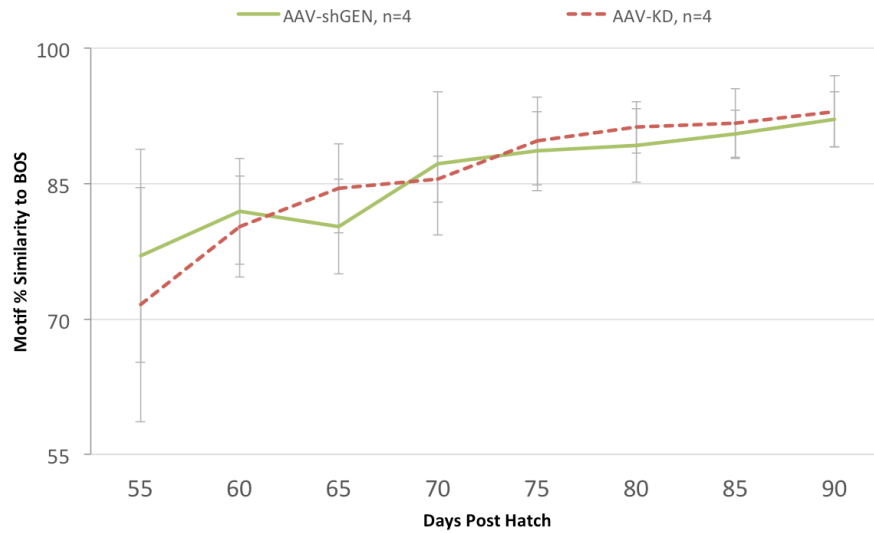


C



A) Similarity between pupil and tutor motifs across sensorimotor learning in 6 non-injected pupils. Each line represents data from a single bird. Lines of the same color indicate that birds shared the same tutor. B) Similarity between pupil and tutor motifs across sensorimotor learning in 4 AAV-KD birds and 4 AAV-shGEN birds. Each line represents data from a single bird. Solid lines represent AAV-shGEN birds; dashed lines represent AAV-KD birds. Lines of the same color indicate that birds shared the same tutor. C) Similarity between pupil and tutor motifs across sensorimotor learning in 6 non-injected birds, as well as 4 AAV-KD birds and 4 AAV-shGEN birds showed in A) and B). Mean score from each group is plotted. Upper and lower whiskers represent SE. AAV-KD treated birds (red dashed line) have significantly lower scores than those of AAV-shGEN treated birds (green solid line) and the non-injected group of birds (black solid line) after 70d. AAV-shGEN and non-injected groups do not exhibit any significant difference from each other. Two asterisks: $p < 0.005$; one asterisk: $p < 0.05$, unpaired one-tailed bootstrap.

Figure 3-8: Quantification of motif similarity of pupil song to its own adult song.



Similarity of motifs sung at different ages to the mature 90d song from 4 AAV-KD birds and 4 AAV-shGEN birds. Mean score from each group is plotted. Upper and lower whiskers represent SE. AAV-KD treated birds (red dashed line) do not differ significantly from AAV-shGEN treated birds (green solid line) throughout the sensorimotor phase ($p > 0.05$, unpaired one-tailed bootstrap).

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Appendix 1: Differential FoxP2 and FoxP1 expression in a vocal learning nucleus of the developing budgerigar

Osceola Whitney, Tawni Voyles, Erina Hara, QianQian Chen, Stephanie A. White and Timothy F. Wright

Abstract

The forkhead domain FoxP2 and P1 transcription factors have been implicated in several cognitive disorders with language deficits, notably autism, and have been shown to play a critical role in learned vocal motor behavior within many vertebrate species, including songbirds and humans. However, the neurodevelopmental expression patterns of *FoxP2* and *FoxP1* in a species with lifelong vocal learning abilities are unknown. Like humans, budgerigars (*Melopsittacus undulatus*) learn new vocalizations throughout their entire lifetime. Like songbirds, budgerigars have distinct brain nuclei for vocal learning, which include the magnocellular nucleus of the medial striatum (MMSt), a basal ganglia region that is considered developmentally and functionally analogous to Area X in songbirds. Here we used *in situ* hybridization and immunohistochemistry to investigate *FoxP2* and *FoxP1* expression in the MMSt of juvenile and adult budgerigars. We found that FoxP2 mRNA and protein expression levels in the MMSt were lower than that of the surrounding striatum throughout development and adulthood. In contrast, for FoxP1 mRNA and protein we found an elevated MMSt/striatum expression ratio as birds matured, regardless of their sex. These results show that the life-long vocal plasticity seen in budgerigars is associated with persistent low-level *FoxP2* expression in the budgerigar MMSt and suggests the possibility that *FoxP1* plays an organizational role in the neural development of vocal motor circuitry. Thus, developmental regulation of the *FoxP2* and *FoxP1* genes in the vocal control region of the basal ganglia appears essential for vocal learning in a range of species that possess this relatively rare trait.

Introduction

Increasing evidence suggests that the underlying genetic mechanisms for vocal learning are shared between divergent taxa such as humans and several evolutionary lineages of birds. The neurogenetic basis for vocal learning is not understood completely, but there is clear evidence for the critical role of the basal ganglia expression of the P subfamily of forkhead box transcription factors, particularly FOXP2 and FOXP1 (Scharff and White, 2004; White et al., 2006; Bolhuis et al., 2010; White, 2010). Activity of the *FOXP2* gene during human embryonic brain development is necessary for the organization of cortical and basal ganglia structures involved in sensorimotor integration and fine orofacial motor control. Mutations in this gene in humans produce speech and language pathologies, and neuroanatomical abnormalities, notably in a striatal region of the basal ganglia (Vargha-Khadem et al., 1998; Lai et al., 2001; Watkins et al., 2002; Belton et al., 2003; Lai et al., 2003; MacDermot et al., 2005). Similar to FOXP2, FOXP1 expression is linked to the development of the CNS and to organogenesis (Ferland et al., 2003; Tamura et al., 2003; Jepsen et al., 2008). Moreover, specific mutations and altered *FOXP1* expression levels were found in patients with general cognitive dysfunctions, including intellectual disability and autism spectrum disorders, along with speech related impairments (Hamdan et al., 2010; Horn et al., 2010; Bacon and Rappold, 2012; Chien et al., 2013; Le Fevre et al., 2013; Tsang et al., 2013).

The avian homologs of the *FoxP* transcription factors appear to regulate neural development and plasticity underlying vocal learning abilities in zebra finch songbirds and possibly other avian vocal learners. *FoxP2* and *FoxP1* show overlapping expression in the basal ganglia of both songbirds and parrots, including a striatal subregion that is necessary for vocal learning in both species (Haesler et al., 2004; Teramitsu et al., 2004). In zebra finches, *FoxP2*

expression in this striatopallidal subregion for vocal learning (Area X) peaks late during sensory motor learning, which suggests a positive association with long-term behavioral consolidation (Haesler et al., 2004). Furthermore, during juvenile sensorimotor learning and adulthood in zebra finches, levels of *FoxP2* mRNA in the striatal vocal control region decrease as birds produce a variable “practice” song that is thought to facilitate vocal motor learning (Teramitsu and White, 2006; Teramitsu et al., 2010). Otherwise, FoxP2 levels remain elevated during singing of stereotyped songs by males. The extent of FoxP2 mRNA and protein downregulation in the striatal vocal control nucleus is related to the amount of singing (Teramitsu and White, 2006; Miller et al., 2008) and is associated with the co-regulation of thousands of genes (Hilliard et al., 2012). Knockdown of *FoxP2* expression via lentivirus-mediated RNA interference in Area X of zebra finches at the onset of sensorimotor learning and continuing into adulthood or during adulthood only resulted in poor learning (Haesler et al., 2007; Murugan et al., 2013), decreased dendritic spine density (Schulz et al., 2010), and abolished dopaminergic (D1R) modulation of vocal variability (Murugan et al., 2013). These investigations in songbirds suggest that *FoxP2* regulates transcription that is associated with structural changes in the basal ganglia that generate vocal variability. FoxP1 in the adult zebra finch brain circuit for vocal control is thought to be involved in the formation of circuits for learned vocal control since its expression closely matches this circuit’s well-known sexual dimorphism (Haesler et al., 2004; Teramitsu et al., 2004).

The Passeriformes, most notably zebra finches (*Taeniopygia guttata*), are the dominant animal model for investigating vocal learning, and such studies have provided essential contributions to understanding of the neural mechanisms, development and evolution of vocal learning with respect to the *FoxP* genes (White, 2010; Scharff and Petri, 2011). Yet in this

species of songbird, only males learn song, and they do so only during an early-life critical period, then lose that ability and cannot learn new vocal patterns in adulthood. In humans, both sexes acquire language, and learning a second language after puberty is possible, albeit with more difficulty than before (Ellis, 1994). Parrots (Psittaciformes), another vocal learning avian taxa, have a number of features critical for investigating neurogenetic mechanisms of human speech, and represent a promising alternative model (Nottebohm, 1972). Parrots are well known for their enduring vocal mimicry abilities (Todt, 1975; Farabaugh and Dooling, 1996; Bradbury, 2004); some parrot species will even mimic human vocalizations and other heterospecific sounds when isolated from conspecifics (Pepperberg, 2010). Furthermore, there is mounting evidence from captive flocks and wild populations that vocal learning is widespread across parrot species, commonly used by juveniles and adults to acquire and modify their vocal repertoire, and appears to be driven, in part, by the need to acquire new group-specific communication signals after immigration to a new social group (Cruickshank et al., 1993; Wright et al., 2008; Scarl and Bradbury, 2009; Salinas-Melgoza and Wright, 2012). Like humans, both male and female budgerigar parrots (*Melopsittacus undulatus*) appear to be open-ended learners that are capable of using auditory feedback to learn new vocalizations throughout adult life (Heaton et al., 1999; Hile and Striedter, 2000; Dahlin et al., 2014). Also like humans, sensory-motor learning for vocal mimicry begins early in budgerigar life. Developmental studies (Brittan-Powell et al., 1997; Hall et al., 1997) show that at ~3 weeks post-hatch begging calls begin to successively transition into their first adult-like contact calls, typically completed in 2 to 3 weeks. The necessity of auditory feedback and the vocal nuclei for vocal mimicry is first apparent around this period (Heaton and Brauth, 1999).

Homologous neural substrates for vocal learning are found in humans, songbirds, and

parrots, including the basal ganglia (Figure A1-1A; Hall et al., 1999; Jarvis and Mello, 2000; Petkov and Jarvis, 2012). Behavioral phenotype differences between close-ended vocal learning songbirds, like the zebra finch and open-ended vocal learning parrots could arise from neurogenetic differences in their basal ganglia center for vocal control.

Here we use budgerigars (*Melopsittacus undulatus*), an open-ended learning species, to test the role of FoxP2 and FoxP1 in long-term behavioral consolidation and/or maturation of the vocal control circuitry. Specifically we examine in budgerigars whether, as found in zebra finches, the expression of *FoxP2* and *FoxP1* are developmentally regulated, and whether their expression is sexually dimorphic. We use *in situ* hybridization and immunohistochemistry to detect FoxP1 and FoxP2 mRNA and protein expression in the brains of juvenile and adult budgerigars of both sexes. We assess young budgerigars during the following 4 distinct developmental periods that coincide with distinct behavioral stages: (1) at the start of their development of “transitional” immature calls beginning ~20 days post hatch; (2) shortly after fledging ~35 days, around which time these birds produce their first adult-like contact call; (3) ~60 days when these birds typically join their first social group; (4) during adulthood, a period during which birds continually learn novel group specific calls (Figure A1-1B).

Methods and materials

Animals and acoustic recording

The budgerigars used for this study were from our breeding colony at NMSU and maintained on a natural light dark cycle, with ad libitum access to food and water. We used a total of 45 budgerigars, 33 at three developmental timepoints, 11 each at 20, 35, and 60 days old (D). In addition, we used 12 adult male and female budgerigars that were all >D120. All of the birds were used for in situ hybridization, 5-6 birds from these age groups were used for immunohistochemistry. All budgerigars were euthanized within a two-day window of reaching the targeted developmental time periods. Of these birds, 18 juveniles were male, 14 juveniles were female, 6 adults were male and 6 adults were female, as determined by sex genotyping using PCR (Pease et al., 2012). Sex genotyping was inconclusive for 1 D60 bird. The birds were individually housed overnight in lab-constructed sound attenuation chambers, the following morning recorded for 2 hours after lights-on at 6 AM, and then immediately euthanized. These birds were acoustically recorded using microphones linked to an 8 channel mixer with digital output to a Windows 7 based PC running Sound Analysis Pro Software (Tchernichovski et al., 2000). The computer digitally captured continuous recordings of all sound events from the chambers. The captured files were then visually inspected using spectrogram analysis, filtered for bird vocalization events, and quantified using Raven Pro software (Cornell Ornithology Lab, Ithaca, NY). Within the 2-hour period of observation most birds in the D35, D60 and D120 groups did not vocalize and no bird in these groups had more than 2 short vocalization events (warbles or contact calls). Some of the D20 birds did vocalize. Five of these birds produced 1381, 322, 210, 95 and 11 vocalizations each, which all contained a mix of warble and call-like

elements. We noted that the call like elements resembled the “transitional” patterned food begging calls found in budgerigars at the earliest stage of sensory-motor learning.

Tissue preparation

Immediately after being acoustically recorded for at least 2 hours in the morning, the birds were weighed and then euthanized via isoflurane inhalation. The whole brain was extracted within 5 minutes and flash frozen using liquid nitrogen. The brain was then stored at -80° C and later sectioned at -20° C using a Leica CM1850 cryostat microtome (Leica Microsystems, Buffalo Grove, IL). Sections of 20 µm were then mounted onto positively charged glass microscope slides (Fisher Scientific, Waltham, MA, #12-550-20) in 7 replicate series. One series was stained with thionin to enable identification of neuroanatomical structures and to help guide localization of the protein expression patterns for FoxP1 and FoxP2 in the MMSt (magnocellular nucleus of the medial striatum) while referencing the budgerigar brain atlas (<http://www.brauthlab.umd.edu/atlas.htm>). Briefly, this staining procedure involved a series of 1-2 minute slide baths in decreasing concentrations of ethanol, 1.5 min in thionin stain, and a water rinse followed by 2 min baths in increasing concentrations of ethanol. Slides were then dipped in xylenes (Sigma-Aldrich, St. Louis, MO, #534056) for 10 minutes, coverslipped with DPX Mountant (Sigma-Aldrich, St. Louis, MO, #06522) and left to dry overnight. The remaining slides were stored at -80° C until analyzed further using *in situ* hybridization and immunohistochemistry.

In situ hybridization and analysis

In situ hybridizations were performed using riboprobes as described previously (Teramitsu et al., 2004; Chen et al., 2013). The probes were designed to hybridize to the 3' region of zebra finch *FoxP1* and *FoxP2*. The *FoxP2* probe corresponded to bp 1870-2127 in budgerigar *FoxP2* coding sequence (GenBank# AY466101.1) and the *FoxP1* probe corresponded to 1731-2035 bp in a predicted budgerigar *FoxP1* coding sequence (NCBI RefSeq XM_005149417.1). The zebra finch *FoxP2* 3' probe and *FoxP1* 3' probe show 98.8% and 97.4% coding sequence identity to their corresponding budgerigar *FoxP2* and *FoxP1* 3' regions, respectively. In contrast, the *FoxP2* 3' probe was only 63.6% identical to budgerigar *FoxP1* sequence and the *FoxP1* 3' probe was also only 63.1% identical to budgerigar *FoxP2* at the coding sequence level. The pattern of expression we found in budgerigars with the *FoxP1* and *FoxP2* probes was consistent with those reported previously in adult parrots using full-length probes (Haesler et al., 2004). We noted that our zebra finch *FoxP1* 3' probe sequence did not overlap with a different zebra finch *FoxP1* 3' probe (Wada et al., 2006) that did not generate a specific hybridization signal in budgerigar brain. Further, specificity of the antisense probes was determined by the absence of a hybridization signal with the corresponding sense probes. To generate probes, the *FoxP* cDNA fragments were amplified by PCR from the pCR 4-TOPO vector (Invitrogen, Carlsbad, CA) using m13F and reverse primers for subsequent in vitro translation with T3 (antisense probes) or T7 (sense probes) RNA polymerase. To hybridize these probes, thaw-mounted 20 um frozen sections were air-dried at RT for 1 hour, quickly rinsed in 1X PBS, and postfixed with 4% paraformaldehyde, pH 7.4. Following acetylation and dehydration, the tissue slides were prehybridized for 1 hour in an oven at 55° C while coverslipped in solution containing 50% formamide, 1X Denhardt's, 0.2% SDS, 10 mM EDTA (pH 8.0), 200 mM Tris (pH=7.8), 1.5 mM NaCl, 250 µg/ml tRNA, and 25 µg/ml poly A. Slides were then hybridized at 55° C overnight in a similar solution that included 10% dextran sulfate

and [³³P]UTP-labeled RNA probes. Equivalent 8 X 10⁶ counts per minute of riboprobes were loaded on each slide for both *FoxP1* and *FoxP2*. Post-hybridization slides were de-coverslipped and rinsed at 55° C for 15 min in 4X SSC, washed at RT for 2 hours in 2X SSC, treated with RNase A (Sigma) for 30 mins, washed twice in 2X SSC for 15 min each at 37° C, and finally washed for 1 hour in 0.25X SSC at 60° C before dehydration in graded ethanols, air-drying and exposure to autoradiographic film (BioMax MR film; Eastman Kodak, Rochester, NY). Slides were exposed to autoradiographic film for ~1 or 2 weeks for *FoxP1* or *FoxP2*, respectively. Developed films were digitized at 600 dpi using a CanoScan 4400P scanner and software (Canon, Ōta, Tokyo, Japan) controlled by a PC running Windows. Film images produced by the ³³P decay emissions of the probes were consistent in consecutive tissue sections and similar expression patterns were observed in multiple birds, confirming probe specificity. Adobe Photoshop (Adobe Systems, San Jose, CA) was used to measure mean pixel intensities of the areas of interest after saving the digital image in a tiff format, which allowed for 8 bits per sampled pixel or 256 different shades of gray to be analyzed. These values for 2 different sections of each brain region in each hemisphere for each animal were imported into JMP software for statistical analysis (SAS Institute Inc., Cary, NC). One-way ANOVAs and Tukey-Kramer HSD were used to analyze group data.

Immunohistochemistry

Fresh-frozen brain sections containing MMSt and adjoining striatum on microscope slides were used to measure FoxP2 and FoxP1 protein expression. Brain sections were first submerged in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, #P6148) for 5 min and

rinsed with 1X phosphate buffered saline (PBS) 3 times for 5 min each. To block nonspecific binding, tissue was incubated in PBST (1X PBS with 0.3% Triton X-100) with 5% donkey serum (Jackson Immuno, West Grove, PA, #107175) for 1 hour at 4° C. Tissue slides were incubated overnight at 4° C in a PBST/1% donkey serum solution containing the polyclonal goat antibody to FoxP2 (Santa Cruz, Dallas, TX, #sc-21069) at 1:1000, and the polyclonal rabbit antibody to FoxP1 (Abcam, Cambridge, MA, #ab16645) at 1:500. Target specificity of the primary antibody for FoxP2 had been previously verified in zebra finches (Soderstrom and Luo, 2010), while the primary antibody for FoxP1 was previously verified in rats (Bowers et al., 2013). We note that the staining pattern for FoxP2 closely matched that for FoxP1; overlapping confocal images show co-expression of FoxP2 and FoxP1 (see Figure A1-3). Following overnight incubation at 4° C, sections were washed 3 times for 5 min each with 1X PBS, then incubated for 2 hours at room temperature in PBST/1% donkey serum and 1:200 dilutions of two fluorescence-tagged secondary antibodies (Abcam, Cambridge, MA) against goat or rabbit IgG, each with distinct excitation spectra (AlexaFluor 488 nm to detect FoxP2, Alexa Fluor 594 nm to detect FoxP1). Slides were then washed with 1X PBS 3 times for 5 min each and coverslipped using Vectashield with DAPI (excited by 405 nm; Vector, Burlingame, CA, #H-1200) as a counterstain. Slides were stored overnight at room temperature before confocal imaging.

Confocal microscopy and quantification

Fluorescent images of protein expression after immunohistochemistry were captured using a Leica TCS SP5 II Broadband Confocal microscope (Leica, Solms Germany). Cytoarchitectural boundaries were determined using the adjacent thionin stained and FoxP1 and

FoxP2 *in situ* hybridized slides. Coronal sections were imaged with at 40X. Optimal beam settings were used for each channel (405 nm for DAPI, 594 nm for FoxP1, 488 nm for FoxP2). For each channel, images of 3 different tissue sections containing the same brain regions (MMSt and the adjoining striatum) were taken for both brain hemispheres of each animal. These confocal images were converted to an 8-bit gray scale, threshold was manually adjusted, and the image was then made into a binary file. Outliers with a radius of < 3 pixels were removed and cell counts were automatically obtained and manually checked using ImageJ software (NIH, Bethesda, MD). The values obtained from cells counts for four brain sections (two from each hemisphere) of each the MMSt and adjoining medial striatum (MSt) were recorded. All FoxP1 and FoxP2 counts were normalized by DAPI to control for varying cell densities. The counts were then averaged for each individual bird. These individual averages were then used to calculate the MMSt/adjoining medial striatum ratio for each animal. The ratios of FoxP1 and FoxP2 expression passed Shapiro-Wilk normality tests, and were analyzed further using a one-way ANOVA with age group as a fixed factor, followed by a *post hoc* pairwise comparison (Tukey-Kramer HSD). JMP software (SAS Institute Inc., Cary, NC) was used for all statistical analyses.

Results

FoxP2 mRNA and protein expression

FoxP2 mRNA expression appeared to be consistently elevated in the medial striatum (MSt) compared to the hyperpallium and nidopallium across all age groups (Table A1-1; Figure A1-2B, C). Moreover, mesopallial expression of *FoxP2* mRNA appeared to increase with age. Juvenile D20 animals showed low mesopallial expression levels similar to that in the nidopallium, while adults (D>120) had higher mesopallial expression similar to that found in the striatum at this age (Figure A1-2A-C). Further analysis of *FoxP2* mRNA expression in the MSt revealed comparably high levels across all age groups (Figure A1-3A). In the MMSt, *FoxP2* mRNA expression varied across development (ANOVA, $F(3,41)=8.98$, $p<0.001$). MMSt *FoxP2* mRNA expression was low at D20 and in adults compared to D35 ($p=0.003$) and D60 ($p<0.001$). Accordingly, similar *FoxP2* mRNA expression levels were found between D20 and adults, as well as between D35 and D60 (Figure A1-3A). Although *FoxP2* mRNA expression in the MMSt was highest at D35 and D60, the ratio of *FoxP2* mRNA expression in the MMSt expression relative to MSt was below 1 across all age groups (Figure A1-3C). We also found group differences in the *FoxP2* MMSt/MSt expression ratio (ANOVA, $F(3,41)=15.75$, $p<0.001$), with a lower MMSt/MSt ratio found at D20 compared to D35 ($p<0.001$) and D60 ($p<0.001$). The *FoxP2* MMSt/MSt ratio was also lower in adults compared to D35 ($p=0.001$). Some D20 birds produced immature vocalizations within the 2-hour period before sacrifice ($n=5$ of 11), and although the *FoxP2* MMSt/MSt ratio at D20 negatively correlated with the amount of vocal production, this relationship only approached significance (Spearman $\rho = -0.616$, $p=0.057$). Sex

differences in *FoxP2* mRNA in the MMSt/MSt (ANOVA, $F(1)>0.01$, $p=0.984$) and its interaction with age ($F(3)=0.462$, $p=0.710$) were not significant.

We next evaluated whether FoxP2 protein expression also showed patterns of reduced expression in the MMSt during development and adulthood (Figures A1-4 and 5). FoxP2 protein expression in the MSt and MMSt across age groups was comparable to that of *FoxP2* mRNA. However, there were differences between age groups in FoxP2 protein expression in the MSt (ANOVA, $F(3,19)$ Ratio=5.08, $p=0.009$; Figure A1-5A). MSt expression in adults was significantly higher than that of both D20 ($p=0.008$) and D60 ($p=0.040$) birds. Expression in the MSt at D35 did not differ from that of any other groups. A one-way ANOVA with age group as a fixed factor approached significance $F(3,19)=2.84$, $p=0.065$). *Post hoc* tests showed FoxP2 protein expression in the MMSt at D20 was significantly lower than that from adults ($p=0.043$). A direct examination of the degree of downregulation in the MMSt using the ratio of FoxP2 protein expression in the MMSt/MSt found no significant differences between the ratios at each age group (ANOVA, $F(3,19)=0.067$, $p=0.580$; Figure A1-5C). Thus, we find that similar to *FoxP2* mRNA, FoxP2 protein expression in the MMSt remains lower than that in the surrounding medial striatum throughout periods of learning in both juveniles and adults, albeit with a slight increase in overall protein levels as birds reach adulthood. A main effect for sex on MMSt/MSt FoxP2 protein expression and its interaction with age was not significant (ANOVA, $F(1)=0.072$, $p=0.792$, and $F(2)=0.229$, $p=0.798$).

FoxP1 mRNA and protein expression

We observed increased FoxP1 *mRNA* expression in the mesopallium and striatum relative to nidopallial and hyperpallial brain regions across all age groups (Table A1-1; Figure A1-2D, E). Although *FoxP1* mRNA expression in the MSt appeared to increase over development, these differences did not reach statistical significance (ANOVA, $F(3,41)=1.43$, $p=0.246$; Figure A1-3B). However *FoxP1* mRNA expression in the MMSt was significantly different across the age groups (ANOVA, $F(3,41)=3.23$, $p=0.320$; Figure A1-3B). D20 birds had significantly less expression than birds at D35 ($p=0.048$) and D>120 ($p=0.046$). Moreover, the ratio of *FoxP1* mRNA expression in the MMSt relative to the MSt was significantly lower at D20 compared to all other age groups ($p<0.001-0.007$). *FoxP1* mRNA expression showed no relationship to vocalizing in D20 birds (Spearman $p=0.058$, $p=0.873$). A main effect of sex and its interaction with age for MMSt/MSt *FoxP1* mRNA expression was also not significant (ANOVA, $F(1)=0.004$, $p=0.286$, and $F(3)=1.096$, $p=0.363$).

Comparable differences were found in FoxP1 protein expression between age groups. Significant differences were found in FoxP1 protein expression between age groups in the MSt (ANOVA, $F(3, 19)=10.19$, $p<0.001$), where adult birds had higher expression compared to D60 ($p=0.026$), D35 ($p=0.024$), and D20 birds ($p<0.001$). Furthermore, there was an age group difference in FoxP1 protein expression in the MMSt (ANOVA, $F(3,19)$ Ratio=12.64, $p<0.001$), and post-hoc tests revealed that FoxP1 expression in D20 birds was significantly lower than that of D35 ($p=0.010$), D60 ($p=0.003$) and adult birds ($p<0.001$). D35, D60 and adult birds did not differ from one another (Figure A1-5B). A ratio of FoxP1 expression in the MMSt compared to the MSt showed the degree of FoxP1 protein expression was also different between the age groups (ANOVA, $F(3,19)=7.96$, $p=0.001$; Figure A1-5D). The ratios of D20 birds were significantly lower than that of D35 ($p=0.010$), D60 ($p=0.001$) and adults birds ($p=0.038$). Thus,

in MMSt, FoxP1 mRNA and protein expression appears to increase after D20. We did not find a significant main effect of sex on MMSt/MSt FoxP1 protein expression (ANOVA, $F(1)=0.083$, $p=0.778$), and its interaction with age was also not significant, and $F(2)=0.557$, $p=0.587$).

FoxP2 and FoxP1 co-expression in the MMSt

As expected, most of the cells expressing FoxP1 protein in the MMSt and MSt overlapped with FoxP2 protein expressing cells; exemplar expression is shown in Figure A1-4G, H. To further explore the possibility of a potential interaction between FoxP2 and FoxP1 in the MMSt we analyzed an expression ratio of FoxP2/FoxP1 protein and found a significant difference across age groups (ANOVA, $F(3, 19)=6.429$, $p=0.004$). In D20 bird the FoxP2/FoxP1 protein expression ratio was significantly higher compared to that ratio in birds at D60 ($p=0.004$) and D120 ($p=0.026$). Although the FoxP2/FoxP1 ratio was highest at D20, it remained <1 across the age groups; mean expression was 0.47, 0.39, 0.30, and 0.35 for D20-D>120, respectively (Figure A1-6).

Discussion

We investigated developmental FoxP2 and FoxP1 mRNA and protein expression within a basal ganglia vocal learning nucleus in the budgerigar, a parrot species with open-ended vocal learning. Our results suggest that these genes play a conserved role for vocal learning in evolutionarily diverse species. Moreover, the developmental *FoxP2* expression pattern we observe here in budgerigars differs from that found during zebra finch development, and is consistent with persistent vocal plasticity in budgerigars. The developmental *FoxP1* expression that we observed in the MMSt provides support for its role, as suggested by others, in the development of vocal motor neural circuitry. Thus, the divergent developmental expression patterns we find for *FoxP2* and *FoxP1* suggests the possibility that these genes may have distinct contributions to the processes underlying vocal ontogeny in species with vocal learning.

Functional implications of FoxP2 expression in budgerigars

Previous research in adult and juvenile zebra finches found that downregulation of *FoxP2* in Area X is related to the production of songs that are undirected, e.g. lacking a particular social target (Teramitsu and White, 2006; Miller et al., 2008; Teramitsu et al., 2010); such singing is understood to be a form of vocal practice employed to maintain learned vocalizations (Oliveczky et al., 2005). This singing-dependent downregulation of *FoxP2* could allow for plasticity in vocal-control circuits that may be necessary for trial and error based learning (Teramitsu and White, 2006), and is consistent with a post-organizational role for FoxP2 in the modulation of neural vocal motor circuits for learning. We could not clearly determine whether learned vocal production in fledgling juveniles or adults downregulates FoxP2 expression, as these budgerigars

rarely vocalized during the 2-hour period of observation prior to euthanization. Moreover the relationship between vocal practice and FoxP2 protein expression in D20 birds could not be fully explored here due to a lack of statistical power as only 3 of 6 D20 birds vocalized, and only 2 of these 3 D20 birds produced >11 vocalizations within the 2 hour period before sacrifice. Nonetheless, a role for FoxP2 that is permissive for plasticity in budgerigars during a heuristic learning approach is suggested by our data that found a trend of lower FoxP2 mRNA expression as vocal production increased in D20 birds.

Downregulation of FoxP2 mRNA and protein expression within the MMSt relative to the MSt was seen in both juvenile and adult budgerigars, and both are capable of learning new vocal patterns. We hypothesize that the persistent low level *FoxP2* expression in the MMSt maintains this region in a state that allows for persistent plasticity; thus permitting mature vocal learning circuits to encode the necessary motor patterns to produce learned vocalizations. If true, then perhaps the extent of vocal learning in budgerigars correlates with the low level of FoxP2 expression in the MMSt. Although it is beyond the scope of this study, we are pursuing the question of whether vocal modification in the budgerigar may be influenced by social contexts such as group membership status (novel or stable), and its potential to influence FoxP2 expression. We should point out here that we are not proposing that FoxP2 is not necessary for vocal learning. Rather, we think our results show that downregulation of this key gene in the MMSt allows for the vocal plasticity seen in both juvenile and adult budgerigars. That is, downregulation of the *FoxP2* gene, which functions as a transcriptional regulator of a suite of other genes (Hilliard et al., 2012), is not equivalent to a gene that is not functioning in vocal learning. Our finding is consistent with results from zebra finches showing that downregulation

of *FoxP2* during undirected singing is associated with greater plasticity in song (Teramitsu and White, 2006).

Previous studies in budgerigars suggested that FoxP2 expression would be elevated in the MMSt relative to the adjoining striatum during early vocal learning periods and then decline as birds entered adulthood, as was found in developing zebra finches (Teramitsu et al., 2004; Haesler et al., 2004). Such regulation in zebra finches suggested a role for FoxP2 in the formation of circuits for learned vocalizations. Developmental expression studies in human brain also support a role for FoxP2 in the development of motor-related circuits (Lai et al., 2003). One explanation for the developmental FoxP2 expression differences between zebra finches and budgerigars, suggests an alternative, though not mutually exclusive, role for FoxP2. That is, the upregulation of *FoxP2* in D35 and D50 zebra finches may be related to long-term consolidation within the neural circuits underlying a specific behavioral performance, e.g. the crystallization of a stereotyped song. We found that *FoxP2* mRNA expression was increased at D35 and D60 compared to D20 and adults, but this expression did not equal or exceed the surrounding striatum at any developmental timepoint we observed. Since budgerigars are open-ended vocal learners they may experience this crystallization to a lesser degree; thus there may be no point in their development during which FoxP2 expression is upregulated in the MMSt relative to the MSt. This hypothesis is consistent with a finding in mice, showing that FoxP2 regulates gene expression crucial for modulating synapse formation (Sia et al., 2013).

We note that as found in humans, differences in learning ability between young and old adults could reflect difference in gene expression (Spieler and Balota, 1996; Lu et al., 2004), including potential differences in FoxP2 expression at these ages. Further investigation of this issue could also use virally mediated gene transfer to overexpress FoxP2 in the MMSt of juvenile

budgerigars and test for its potential effects on adult vocal learning. Also of potential significance in open-ended learning species such as the budgerigar, is whether labeling intensity of FoxP2 expressing neurons correlates with neurogenesis. An exponential decrease in FoxP2 protein expression in Area X of zebra finches was found between young birds at D35 and D50 compared to adults and intensely labeled cells overlapped significantly with BrDU labeled new neurons (Thompson et al., 2013). Measuring intensity levels was beyond the technical scope of the immunofluorescent procedure used in this study.

The role of FoxP1 expression and interactions with FoxP2

The FoxP1 mRNA and protein expression ratio for the MMSt relative to the MSt was <1 in the D20 birds, but increased significantly in D35, D60, and adult birds, showing that FoxP1 in the MMSt is being upregulated relative to the adjoining striatum as birds matured. This finding is similar to those found in vocal learning songbirds, where *FoxP1* mRNA expression in zebra finches was also upregulated in Area X relative to the MSt in juvenile and adult male birds. However in zebra finches, Area X/outlying striatum *FoxP1* mRNA expression appeared to peak in younger (D35) birds, whereas in budgerigars, this FoxP1 mRNA and protein ratio was highest at D60. This expression pattern was unlike that of FoxP2 and suggests that upregulation of FoxP1 expression in the MMSt (or Area X) plays a role in the development and adult function of basal ganglia circuitry that is required for vocal plasticity. A similar role for FoxP1 has been described in developing mouse brain (Ferland et al., 2003). Differences in the timing of peak FoxP1 expression in the motor circuitry could reflect differences in the corresponding rates of maturation in different species.

FoxP1 may interact with FoxP2 and other genes in the FoxP family to regulate genes involved in the development and maintenance of vocal learning circuits. FoxP2 and FoxP1 act in cooperation to regulate development of mouse lung and esophageal tissues (Shu et al., 2007) so perhaps these genes cooperate to establish and modify connections in the brain as well. In songbirds, *FoxP2* and *FoxP1* are co-expressed in the striatum (Chen et al., 2013). Here we found in budgerigars overlapping FoxP2 and FoxP1 protein expression in the same MMSt cells and that the ratio of MMSt FoxP2/FoxP1 protein expression was highest in nestling birds, significantly decreasing as birds aged. The decreasing ratio was due to a prodigious increase in FoxP1 protein expression as birds aged. These results suggest that a primary role for FoxP1 expression, during early development and not later, could be interacting with FoxP2 for the cooperative regulation of gene expression.

In rodents FoxP2 and FoxP1 expression overlaps with FoxP4 (Takahashi et al., 2003) and all three of these FoxP members have the ability to heterodimerize with one another (Li et al., 2004). Moreover, a knockout of FoxP4 in developing mice resulted in the deterioration of Purkinje cells in the cerebellum (Tam et al., 2011). Since Purkinje cells aid the coordination and timing of learned sequences of motor movements, perhaps a similar coordination of vocal motor movements used in vocal learning species also use activity of the FoxP4 transcription factor. A preliminary investigation found that in zebra finch song nuclei HVC (a proper name) and RA (robust nucleus of arcopallium), FoxP1 and FoxP4 were co-expressed (Medoza et al., 2013). It is important to note that potential interactions between the FoxP transcription factors may not be shared among all vocal control regions, and thus contribute specialized roles to the maturation and modulation of vocal control circuitry and to the process of vocal imitation. In humans,

individual mutations in FOXP1 and FOXP2 result in different phenotypes, each characterized by a unique set of deficits (Bacon and Rappold, 2012).

Lack of sex differences but a mesopallial increase in FoxP expression

Unlike in the zebra finch, where *FoxP2* and *FoxP1* mRNA expression is sexually dimorphic, we detected no differences in FoxP2 and FoxP1 gene expression between male and female budgerigars. This result is consistent with the vocal learning behavior observed in this species, as both sexes have been shown to learn new vocalizations, even as adults (Farabaugh and Dooling, 1996; Hile and Striedter, 2000; Dahlin et al., 2014). Interestingly our data suggest a role for the FoxP genes outside of the striatum. We found a striking difference in *FoxP2* and *FoxP1* mRNA expression in the mesopallium, where expression gradually increased throughout development into adulthood, in parallel with the acquisition and increase of the vocal repertoire. Although this region of the mesopallium does not contain vocal control nuclei, in general the mesopallium is enlarged in birds with high cognitive abilities, like parrots (Lefebvre et al., 2004; Iwaniuk and Hurd, 2005; Chen et al., 2013). Cognitive complexity in birds may be dependent on mesopallial brain organization and, as our data suggests, its underlying gene activity that includes the FoxP2 and FoxP1 genes.

Conclusions

Vocal learning has evolved independently in various groups of birds and mammals (Petkov and Jarvis, 2012), yet the exact physiological components of this complex behavior are

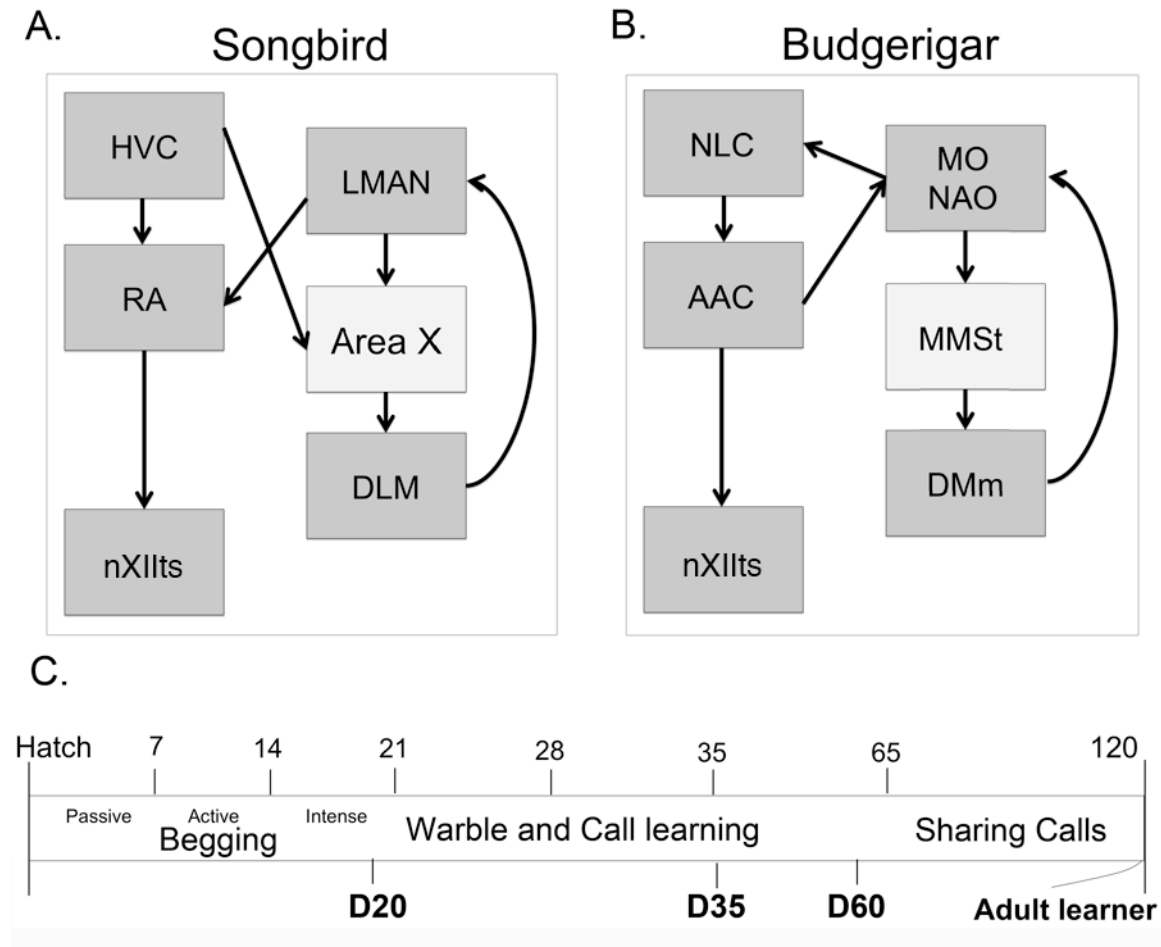
not completely understood. The results from this experiment shed light on some of the neuromolecular mechanisms that allow vocal learning in juvenile and adult animals, and add to the increasing evidence for common neurogenetic mechanisms underlying learned vocal communication. Further investigation of FoxP gene regulation in budgerigars is essential for understanding both developmental and adult-utilized processes for learned vocal communication.

Acknowledgements

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Figures

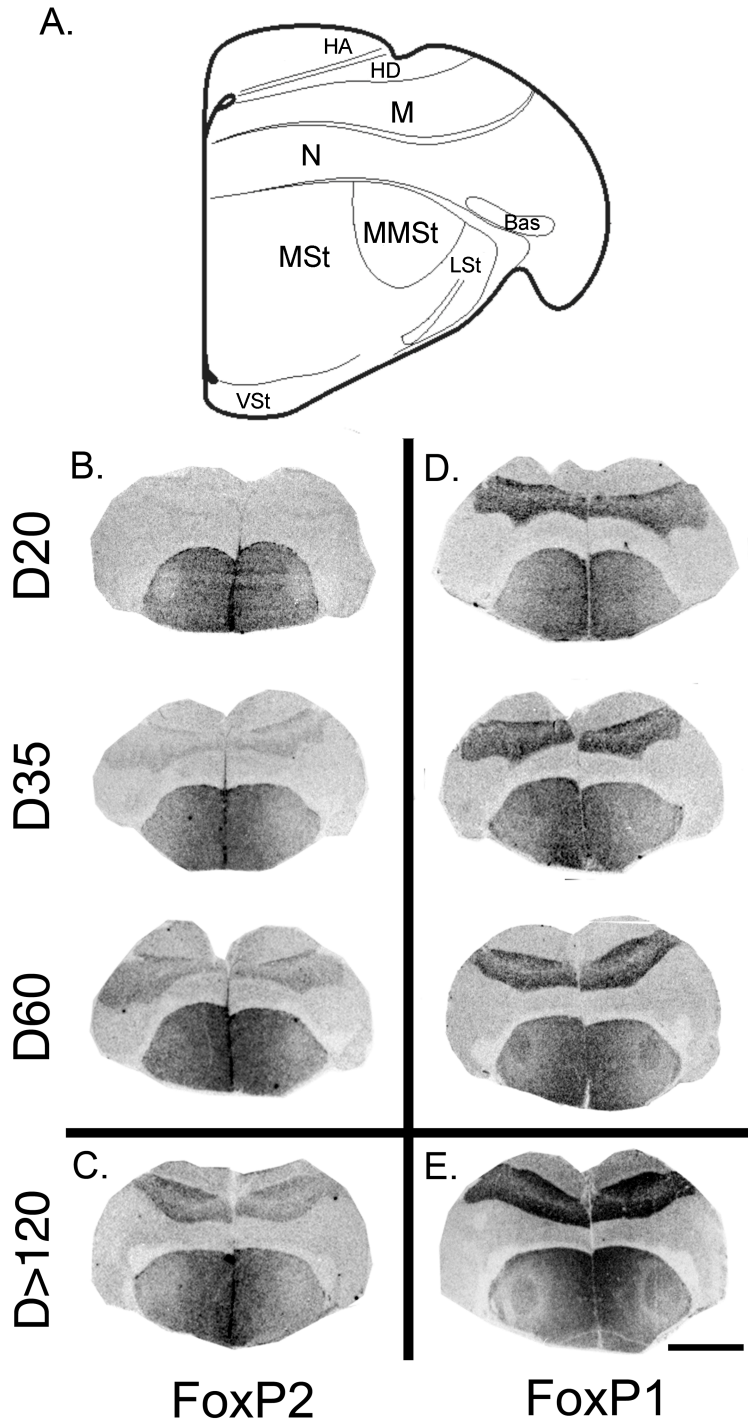
Figure A1-1: Schematic of vocal control nuclei in songbird and budgerigar.



Shown here is a general schematic of interconnected vocal control nuclei in (A) the songbird brain and (B) the budgerigar brain (Nottebohm et al., 1976; Striedter, 1994). Area X and MMSt in the basal ganglia are part of a cortico-basal ganglia-thalamo-cortical loop important for learning acoustic gestures (Petkov and Jarvis, 2012). The songbird and budgerigar CNS via nXIIts projects to the syringeal muscles that produce sound. (C). Vocal development of budgerigars begins after hatch with food begging calls followed by a transitional period beginning ~D13 when immature socially learned vocalizations are first generated. Adult like

vocalizations emerge 5 to 6 weeks after hatch, and at ~D60 birds begin to join social groups and imitate conspecifics (Brittan-Powell et al., 1997; Hall et al., 1999). Vocal learning in new social groups occurs frequently in adults. The present study used birds that were isolated and recorded at the ages shown in bold, D20, D35, D60 and adults D>120. Abbreviations: Songbird: Area X and HVC are used as proper names: DLM, medial portion of the dorsolateral thalamic nucleus; LMAN, lateral magnocellular nucleus of the anterior nidopallium; RA, robust nucleus of the archipallium. Budgerigar: AAc, central nucleus of the anterior arcopallium; MO, oval nucleus of the mesopallium; MMSt, magnocellular nucleus of the medial striatum; NAO, oval nucleus of the anterior nidopallium; DMm magnocellular nucleus of the dorsomedial thalamus; nXIIts, tracheosyringeal motor nucleus, a portion of the 12th (hypoglossal) nucleus.

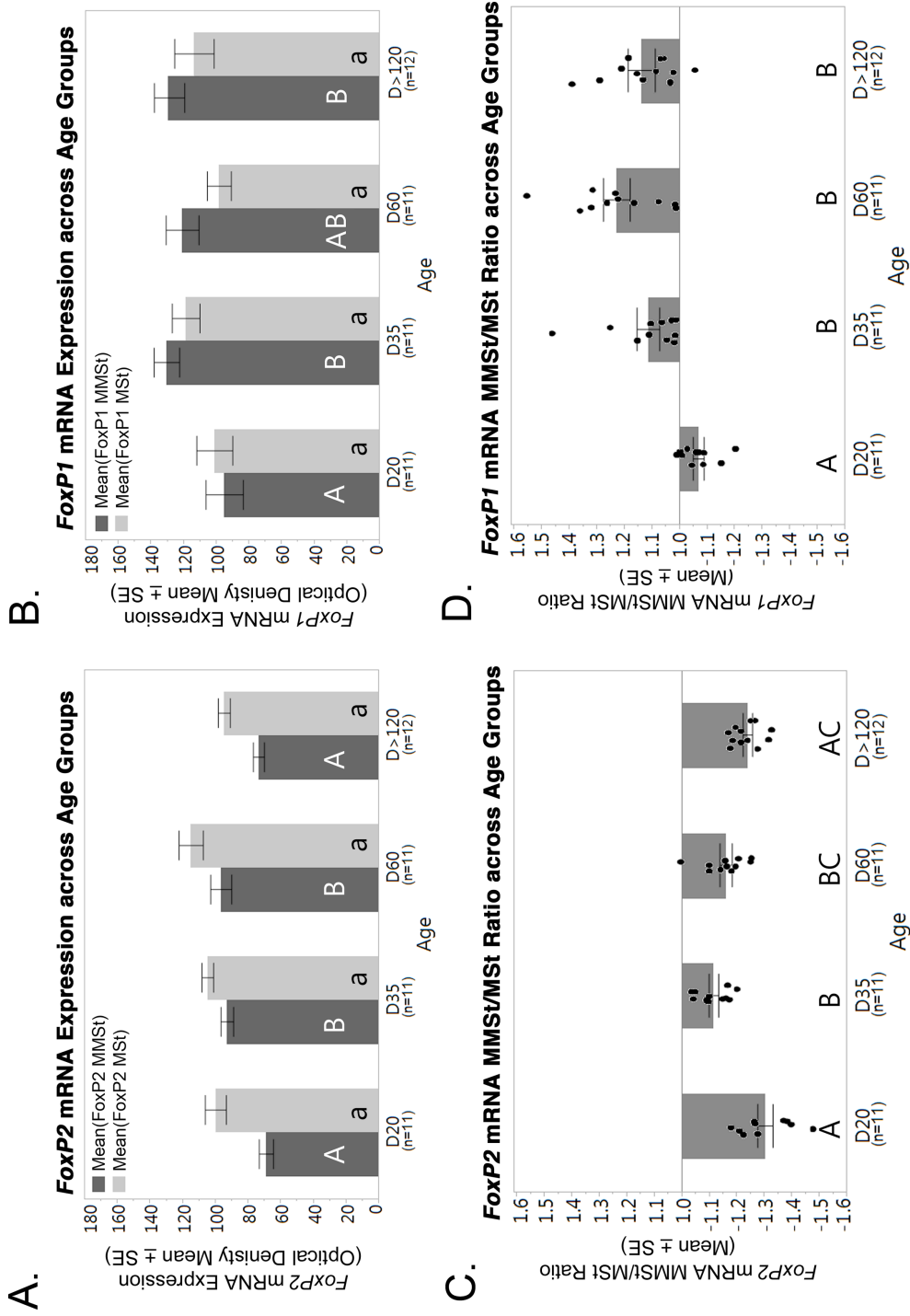
Figure A1-2: FoxP2 mRNA expression in male budgerigar brain at different developmental stage.



A) Location of the MMSt and adjoining striatum (MSt) in a schematic section from the budgerigar brain atlas at <http://www.brauthlab.umd.edu/atlas.htm> (Also see: Reiner et al., 2004).

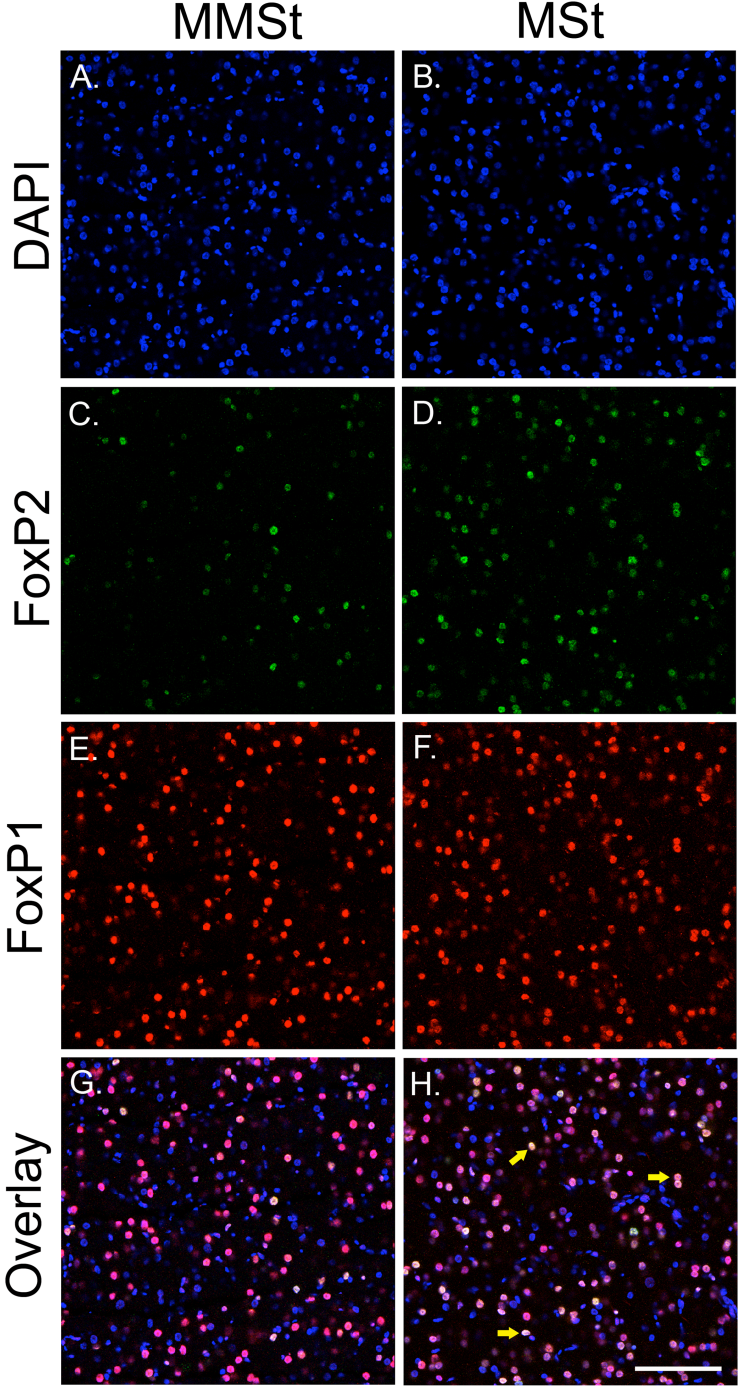
B) *In situ* hybridized *FoxP2* mRNA in which the MMSt can be found at B) D20, D35, D60, and C) in adults, D>120 (all birds are male). Sections of similar male brains show *in situ* hybridized *FoxP1* mRNA in D) and E). "*" denotes the adjoining medial striatum where gene expression measurements were obtained. Scale bar in E) = 4 mm. Abbreviations: HA, Hyperpallium, apical; HD, Hyperpallium, densocellular; M, Mesopallium; N, Nidopallium; Bas, Basorostral pallial nucleus; MMSt, Magnocellular nucleus of the medial striatum; MSt, Medial striatum; LSt, Lateral striatum; VSt, Ventral striatum.

Figure A1-3: *FoxP2* mRNA expression quantification across age groups.



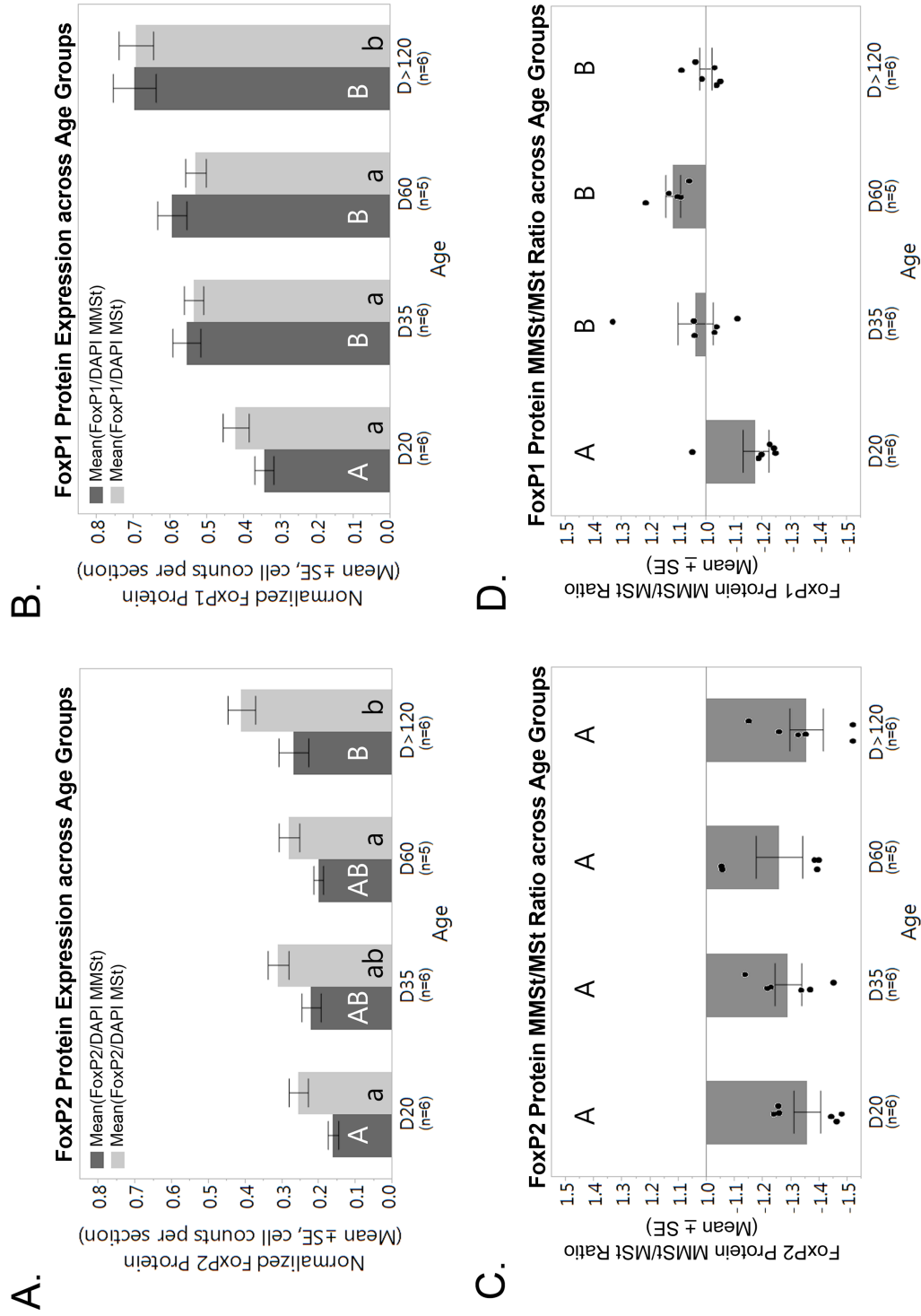
A) *FoxP2* mRNA expression in the MMSt (dark grey) and MSt (light grey) across age groups. No significant differences were found between groups in the MSt. B) *FoxP1* mRNA expression in the MMSt and MSt across age groups. No significant differences were found between age groups for both the MSt. *FoxP1* mRNA expression in the MMSt was significantly lower at D20 compared to D35 and D>120. (C, D) *FoxP2* and *FoxP1* mRNA expression ratios (MMSt/MSt) show significant differences between age groups ($p < 0.05$). Points in C) and D) represent individual birds. For all graphs, significant mRNA expression differences in the MMSt, MSt, (A, B, respectively) or a ratio thereof (C, D), between the four age groups is denoted with different letters. Bars not connected by the same letter are significantly different ($p < 0.05$). The letter case is used to denote significant differences between age groups separately for the MMSt (uppercase) and MSt (lowercase). Error bars = SE.

Figure A1-4: Confocal images (40X) used for detection of FoxP2 and FoxP1 within the MSt and MMSt.



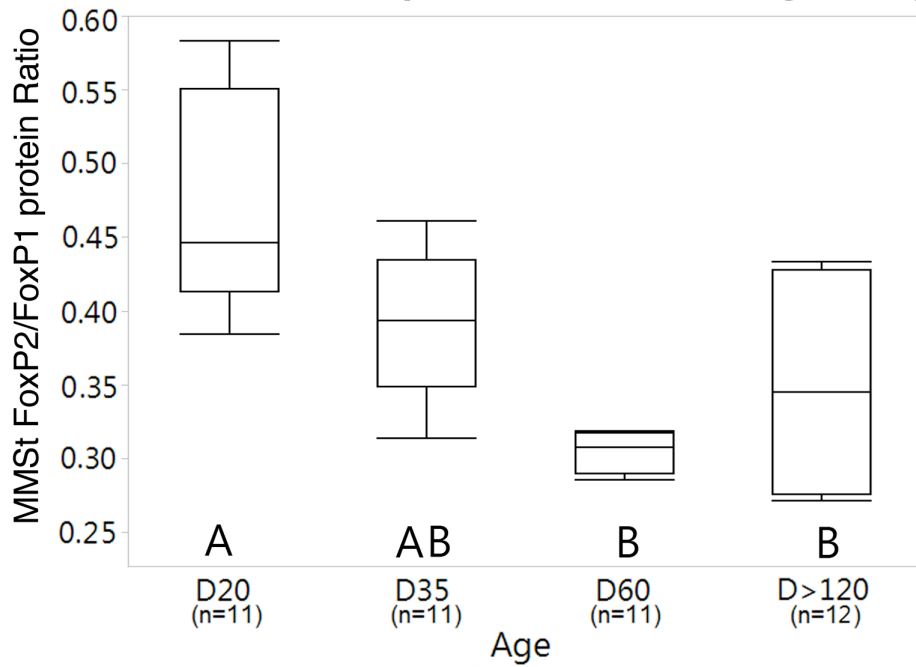
Example images are shown from a female animal at D35. From top to bottom: A) and B) DAPI stained cells in 405 nm within the MMSt and MSt; C) and D) FoxP2 protein expressing neurons in 488 nm within the MMSt and MSt; E) and F) FoxP1 expressing neurons in 594 nm in the MMSt and MSt; G) and H) an overlay of the 3 channels, note in G) that many of the cells expressing FoxP1 also express FoxP2 (yellow arrows). Scale bar in H = 50 μ M

Figure A1-5: FoxP2 protein expression quantification across age groups.



A) DAPI normalized FoxP2 protein expression across age groups in the MMSt (dark grey) and MSt (light grey). Significant differences between age groups in the MMSt and MSt. are shown with bars not connected by the same letter ($p < 0.05$). B) DAPI normalized FoxP1 protein expression across age groups in the MMSt (dark grey) and MSt (light grey) show significant differences in D20 and adult birds with bars not connected by the same letter ($p < 0.05$). C) FoxP2 and D) FoxP1 MMSt/MSt protein expression ratios across age groups. No significant differences in FoxP2 expression ratios were found between groups. Ratios were < 1 for all age groups. D) MMSt/MSt FoxP1 protein expression ratios were significantly lower at D20 ($p < 0.05$). Points in C) and D) represent individual birds. Error bars = SE.

Figure A1-6: Ratio of FoxP2/FoxP1 protein expression in the MMSt across age group.



Box plots show a ratio of FoxP2/FoxP1 protein expression in the MMSt. FoxP expression is DAPI normalized. Top and bottom whisker lines, and box line represent maximum, minimum and median median values, respectively.

Tables

Table A1-1: Mean optical density values of *FoxP* mRNA expression in budgerigar brains normalized to background.

Brain Region	D20		D20		D35		D35		D60		D60		Adult		Adult	
	<i>FoxP1</i>	<i>FoxP2</i>	<i>FoxP1</i>	<i>FoxP2</i>	<i>FoxP1</i>	<i>FoxP2</i>	<i>FoxP1</i>	<i>FoxP2</i>	<i>FoxP1</i>	<i>FoxP2</i>	<i>FoxP1</i>	<i>FoxP2</i>	<i>FoxP1</i>	<i>FoxP2</i>	<i>FoxP1</i>	<i>FoxP2</i>
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
Hyperpallium	57	58	36	37	71	74	52	46	77	80	57	55	78	77	61	68
Mesopallium	119	128	48	47	143	146	71	77	147	146	78	78	152	158	99	103
Nidopallium	49	54	50	42	49	49	42	33	60	53	48	47	59	54	53	60
Basorostral pallial nucleus	18	24	33	39	21	22	24	18	24	19	28	31	22	28	18	27
Medial striatum (MSt)	106	93	92	114	118	122	100	118	107	95	127	110	111	119	91	102
Magnocellular nucleus of the medial striatum (MMSt)	100	87	64	78	130	133	90	101	127	118	104	94	130	139	70	79

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Appendix 2: Neural FoxP2 and FoxP1 expression in the budgerigar, an avian species with adult vocal learning

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Abstract

Vocal learning underlies acquisition of both language in humans and vocal signals in some avian taxa. These bird groups and humans exhibit convergent developmental phases and associated brain pathways for vocal communication. The transcription factor *FoxP2* plays critical roles in vocal learning in humans and songbirds. Another member of the forkhead box gene family, *FoxP1* also shows high expression in brain areas involved in vocal learning and production. Here, we investigate *FoxP2* and *FoxP1* mRNA and protein in adult male budgerigars (*Melopsittacus undulatus*), a parrot species that exhibits vocal learning as both juveniles and adults. To examine these molecules in adult vocal learners, we compared their expression patterns in the budgerigar striatal nucleus involved in vocal learning, magnocellular nucleus of the medial striatum (MMSt), across birds with different vocal states, such as vocalizing to a female (directed), vocalizing alone (undirected), and non-vocalizing. We found that both *FoxP2* mRNA and protein expressions were consistently lower in MMSt than in the adjacent striatum regardless of the vocal states, whereas previous work has shown that songbirds exhibit down-regulation in the homologous region, Area X, only after singing alone. In contrast, *FoxP1* levels were high in MMSt compared to the adjacent striatum in all groups. Taken together these results strengthen the general hypothesis that *FoxP2* and *FoxP1* have specialized expression in vocal nuclei across a range of taxa, and suggest that the adult vocal plasticity seen in budgerigars may be a product of persistent down-regulation of *FoxP2* in MMSt.

Introduction

Vocal learning is a phylogenetically rare trait found in relatively few evolutionary lineages including humans and some avian taxa [1,2]. These birds, which include songbirds and parrots, exhibit convergent developmental phases and brain pathways for learned vocal communication with humans [2], highlighting their value as models for investigating the neural and genetic basis of vocal learning.

The transcription factor *FOXP2*, a member of the forkhead box family, plays an important role in human speech. Mutations of this gene cause speech impairments due to poor coordination of orofacial movement [3], and structural and functional abnormalities in various brain regions including the basal ganglia and Broca's area [4,5]. Interestingly, in songbirds, *FoxP2* levels change both developmentally and acutely within the striatal (basal ganglia) vocal control nucleus, Area X, which is critical for vocal learning in songbirds [6-9]. In juvenile male zebra finches, *FoxP2* mRNA expression increases in Area X during the sensorimotor song learning period, and disruption of the gene through shRNA-mediated knockdown disrupts song learning [7,10,11]. When adult males produce songs alone, known as undirected singing, *FoxP2* mRNA expression decreases in Area X compared to baseline levels in non-singing birds [8,9]. Consistent with the mRNA data, both Western blot and immunohistochemistry reveals that FoxP2 protein decreases when birds produced undirected song relative to levels in non-singing birds. [8,12,13].

Another member of the forkhead box gene family, *FoxP1*, is also thought to play a role in brain regions involved in learning and producing vocalizations. *FoxP1* is highly expressed in various song nuclei in songbirds, and the level of expression is similar across different ages and

singing contexts [6-9]. Interestingly, along with general cognitive dysfunction, mutations in *FOXP1* are also implicated in abnormal human speech development [14-19].

Song learning in the predominant songbird models is restricted to males and occurs only during a critical period early in life. In humans, however, both sexes maintain the capacity to learn new words or languages through adulthood. The budgerigar is a small parrot in which both males and females exhibit large vocal repertoires and the ability to learn new contact calls in adulthood [20-22]. *FoxP1* and *FoxP2* mRNAs are expressed in the striatal vocal learning nucleus, magnocellular nucleus of medial striatum (MMSt) of the budgerigar [6], however, it remains unclear whether vocal behavior acutely alters FoxP expression as it does in zebra and Bengalese finches [9].

Here we investigate the mRNA and protein expression of *FoxP2* and *FoxP1* in MMSt of budgerigars in different vocal states (vocalizing either in the presence of females or alone, and non-vocalizing) and compared these patterns to those in non-singing zebra finches. If *FoxP2* expression in MMSt is behavior-driven as in Area X of the male zebra finch (low *FoxP2* expression when they sing alone), then low expression is expected in MMSt when budgerigar males produce vocalizations alone. Alternatively, if the persistent vocal plasticity in budgerigars relies on continually lowered levels of *FoxP2* in MMSt, then we expect low levels in all groups. Since there is no evidence from previous studies that the expression pattern of *FoxP1* is behaviorally driven, we predict high *FoxP1* expression in MMSt across vocal states as in other avian models.

Materials and Methods

Subjects

Eighteen adult male budgerigars (*Melopsittacus undulatus*) and four adult male zebra finches (*Taeniopygia guttata*) from our breeding colony or a local supplier were used in this experiment. Six adult female budgerigars were used to stimulate vocal behavior. They were group-housed with other adult conspecifics on a 12L:12D hour photoperiod with food and water *ad libitum*. All the experimental procedures were approved by New Mexico State University, Animal Care and Use Committee (protocols 2010-001 and 2013-030).

Behavior

Adult male budgerigars were randomly assigned to the following three different vocal states: i) female directed vocalizing (n=6), ii) undirected vocalizing (n=6), and iii) non-vocalizing (n=6). For the non-vocalizing group, we used birds that produced less than 8 total individual vocalizations, which included contact calls (0-2 calls) and other types of vocalizations (0-6 calls) during the recording sessions. Previous studies in zebra finches typically quantified only the amount of singing and did not include other calls (S.A. White, per obs), therefore their non-singing group also sometimes produced non-learned vocalizations. Therefore, our definition of “non-vocalizing group” is consistent with previous studies. As detailed below in the Results, some birds from each group produced “warble songs”, another type of learned vocalization noted for its complexity and variability [23]. We classified warble songs into bouts using previously established criteria [24]: a bout should i) consist of three different elements and ii) be more than

1 second long. If the warble is more than 10 seconds long, every 10 seconds counts as a separate warble bout. Since the duration of warble bouts classified in this way varies, we also counted the number of individual elements in each warble song [23]. For zebra finches, all of the males were non-singing (n=4); they did not produce any songs during the recording session. For the female directed vocalizing group, male budgerigars were moved to individual sound attenuation chambers with a microphone (23 x 25.5 x 48cm) on the morning of recording. Stimulant females were housed in other sound attenuation chambers, which were placed in front of each male assigned to the directed calling group. For undirected and non-vocalizing groups, male budgerigars were housed in individual recording chambers (75x27.5x 28.8cm) two days prior to the recording. On the third day, behavioral observation was performed in the morning. All the observation was between 90-120 minutes after the lights were turned on, and sounds were continuously recorded and digitized using Sound Analysis Pro [25]. All the animals had access to food and water *ad libitum* during the session.

Vocal Counting

All vocalizations from the recordings were manually counted from spectrograms using Raven Pro 1.4 software (Cornell Lab of Ornithology, Ithaca, NY). Recording sessions varied from 90 to 120 minutes. Consequently, we used the rate of vocal element production (number of contact call elements or number of warble song elements divided by total minutes) to analyze the number of vocalizations in the given recording time for our analysis. We also counted the number of bouts of warble following a previous study [24], such that 10 seconds or less of continuous warble was counted as a single bout, while warbles lasting more than 10 second were

classified as 1 bout for each 10 seconds of continuous warble. For budgerigars, we tallied the number of contact call elements, and the number of warble song bouts, and warble song elements in the recording session. No zebra finches produced songs, therefore we did not analyze song rate.

Tissue Preparation

Immediately after the recording session, birds were overdosed with isoflurane and decapitated to dissect their brains. Brains were flash frozen within five minutes on aluminum dishes floated on liquid nitrogen and then stored at -80°C until use. Brains were cryo-sectioned (Leica CM1850, Leica Microsystems, Buffalo Grove, IL) in the coronal plane at $20\ \mu\text{m}$ thickness and thaw-mounted directly on positively charged slides (Fisher Scientific, Waltham, MA) and kept in an -80°C freezer. To enable visualization of key brain regions, some sections were Nissl stained using a series of thionin, alcohol, and xylene washes. Adjacent slides were assigned for *in situ* hybridizations and immunohistochemistry.

In situ hybridization

In situ hybridization was performed using riboprobes as described in Teramitsu et al. [7] except that the *FoxP* cDNA fragments were amplified by PCR from the pCR 4-TOPO vector (Invitrogen, Carlsbad, CA) using m13F and reverse primers. Briefly, sections were prepared for hybridization by fixation (4% paraformaldehyde), acetylation, and incubation of pre-hybridization buffer containing 50% formamide, 1X Denhardt's, 0.2% SDS, 10 mM EDTA (pH

8.0), 200 mM Tris (pH=7.8), 1.5 mM NaCl, 250 μ g/ml tRNA, and 25 μ g/ml polyA. Then sections were hybridized with 33 P-UTP labeled RNA probes over night at 55°C in similar buffer that contain 10% dextran sulfate and 33 P-UTP labeled RNA probes. On the next morning, we performed a series of SSC washes and slides were exposed to Biomax MR films (Eastman Kodak, Rochester, NY). The films were developed with Kodak developer and fixer (Eastman Kodak) for one week for *FoxP1* and two weeks for *FoxP2*.

Zebra finch *FoxP2* and *FoxP1* clones were used in this experiment. We tested probes both from 3' end and middle region of coding sequence and found similar expression patterns. For consistency we used the 3' end probes for both *FoxP2* and *FoxP1* in all of our experiments. For the region of *FoxP2* and *FoxP1* coding sequences covered by these probes, zebra finch and budgerigar (GenBankAY466101.1 and NCBI RefSeq XM_005149417.1) have more than 97% sequence identity. In contrast, budgerigar *FoxP1* and zebra finch *FoxP2* have 63% identity over these regions while budgerigar *FoxP2* and zebra finch *FoxP1* also have only 63% identity. Therefore, cross-hybridization between *FoxP1* probes and *FoxP2* mRNA, and vice versa, is unlikely given our hybridization stringency. Sense probes were used for both *FoxP1* and *FoxP2* as negative controls.

The intensity of *FoxP2* and *FoxP1* expression was quantified from digitized photomicrographs of the x-ray films. Images were opened using Adobe Photoshop (Adobe Systems Inc. San Jose, CA) and were quantified by using the histogram tool to measure the level of signal intensity. Two sections from each hemispheres were quantified, the values averaged, and the average background intensity from outside of brain sections subtracted. To compare the expression of *FoxP2* and *FoxP1* among the groups, we used the ratio of MMS_t intensity divided by intensity of the adjacent area within the striatum (adjacent striatum) to correct for differences

in overall expression level from slide to slide or run to run. Since we used zebra finch clones for our probe, signals were expected to be stronger in zebra finch sections. Therefore, this internal control is critical for cross-species comparisons. During our initial data analysis we examined the distributions for our data, and found that most of them were not normal, nor could they be transformed to normality with the most common transformations. Therefore using JMP software, we performed non-parametric tests (Wilcoxon or Kruskal-Wallis tests), which are robust to deviations from normality and appropriate for small sample sizes. To examine the relationship between the call/warble element rate and gene expression, we ran Spearman's Rho test using JMP software Version 11.0 (SAS Institute Inc., Cary NC).

Immunohistochemistry

Non-contact calling budgerigars (n = 4) and non-singing zebra finches (n = 4) were also examined for FoxP2 and FoxP1 labeled cells with immunohistochemistry. We used sections adjacent to those used for *in situ* hybridization. Sections were fixed with 4% paraformaldehyde for 10 minutes, rinsed three times with 1x PBS for 5 minutes each, incubated in 5% normal donkey serum (Jackson Immuno, West Grove, PA) solution with PBST (1x PBS with 0.3% Triton-X) for 1 hour at 4° C, and then incubated overnight at 4° C in a combination of FoxP1 (Rabbit, 1:500. ab16645. Abcam, Cambridge, MA) and FoxP2 (Goat, 1:1000. sc21069. Santa Cruz, Dulles TX) primary antibodies in humidified slide chambers. Both antibodies are successfully used in avian systems previously [26-28]. Sections were rinsed three times with 1x PBS for 5 minutes each, and incubated with a mixture of Alexa Fluor 488 (Donkey, 1:200. Life Technologies, Carlsbad, CA) and Alexa Fluor 594 (Donkey, 1:200) secondary antibodies for 2

hours at room temperature. Sections were rinsed three times, and coverslipped with Vectashield DAPI (Vector, Burlingame, CA). The same procedure without primary antibodies was performed as a negative control.

For quantification, we used confocal microscope (Leica TCS SP5 II, Leica, Solms, Germany) digital images taken from both left and right hemispheres from two sections. It should be noted that pictures of the adjacent striatum for the IHC analysis were taken from a more medial area than those for the *in situ* hybridization analysis. To count labeled cells for DAPI, FoxP2 and FoxP1, we used Image J (NIH, Bethesda, MD). Images were converted to 8-bit gray scale and made into a binary file that performed partial automatic counting. Cells that were three pixels or greater in size were automatically counted. We then manually adjusted to include labeled cells that were not automatically counted and noise that was incorrectly counted as a labeled cell. Cells were divided by the total number of cells (DAPI) and averaged for each individual animal because of the possible difference in cell density in the areas of interest. These averages then were used to determine the MMSt/Adjacent striatum ratio to correct for differences in florescent level from slide to slide or run to run. Values from budgerigars and zebra finches were compared using Wilcoxon unpaired tests.

Results

Vocal analysis

The number of contact calls and the number of individual elements and bouts of warble songs emitted by male budgerigars were counted and divided by the recording time to obtain vocalization rates. Birds that produced less than 8 total individual vocalizations during recording session were classified as non-vocalizing and retained for analysis. In vocalizing groups, contact call rates (contact calls/minute) varied from 0.03 to 7.89, and there was no significant difference in calling rates between directed and undirected groups when testing with a t-test. (d.f.=5.12, t ratio=-1.39, $p=0.21$). Three birds from the directed vocalizing group and one from non-vocalizing group produced a small number of warble songs (0.01 to 0.22 warble song bouts/minute, and 0.04 to 3.88 warble elements/min).

There was no association between contact call rates and gene expression patterns for either directed (*FoxP1*; Spearman's $\rho=0.08$ $p=0.87$, *FoxP2*; Spearman's $\rho=0.2$ $p=0.70$) or undirected vocalizing groups (*FoxP1*; Spearman's $\rho=-0.37$ $p=0.47$, *FoxP2*; Spearman's $\rho=0.43$ $p=0.40$). Moreover, neither the rate of warble song elements nor of song bouts was correlated with gene expression levels (Warble bout rate: *FoxP1*; Spearman's $\rho=0.20$ $p=0.80$, *FoxP2*; Spearman's $\rho=-0.60$ $p=0.40$. Warble element rate: *FoxP1*; Spearman's $\rho=0.20$ $p=0.80$, *FoxP2*; Spearman's $\rho=-0.60$ $p=0.40$).

FoxP2 mRNA expression in budgerigar MMSt and zebra finch Area X

We observed a lower level of *FoxP2* in MMSt compared to the adjacent striatum in all budgerigar groups (Figure A2-1). The mean ratio with standard error of mean (SEM) for budgerigar directed vocalizing = 0.78 ± 0.03 , budgerigar undirected vocalizing = 0.78 ± 0.03 , and budgerigar non-vocalizing = 0.72 ± 0.03 , whereas non-singing zebra finches exhibited equivalent levels across the striatum (zebra finch non-singing = 1.02 ± 0.04). In budgerigars, the expression gradually increased from MMSt to medial striatum (Figure A2-1). Kruskal-Wallis tests revealed a significant difference among groups in the ratio of striatal vocal control nucleus to adjacent striatum ($\chi^2 = 11.58$, d.f=3, $p=0.01$). We used Wilcoxon tests for *posthoc* pairwise comparisons. These tests revealed that *FoxP2* ratios from zebra finches were higher than those from all budgerigar groups (Figure A2-2, zebra finch non-singing vs. budgerigar directed vocalizing, $p=0.01$; zebra finch non-singing vs. budgerigar undirected vocalizing, $p=0.01$; zebra finch non-singing vs. budgerigar non-vocalizing, $p=0.01$). There was no statistical difference among budgerigar groups (Figure A2-2). Thus expression patterns in the striatal vocal control nucleus differ between species, and budgerigars maintain low *FoxP2* levels in MMSt regardless of the vocalization state.

FoxP1 mRNA expression in budgerigar MMSt and zebra finch Area X

Striatal vocal control nuclei (MMSt and Area X) exhibited a high expression level of *FoxP1* compared to the adjacent striatum (Figure A2-1 and Figure A2-2 mean ratio with SEM for budgerigar directed vocalizing = 1.22 ± 0.03 , budgerigar undirected vocalizing =

1.14 \pm 0.03, budgerigar non-vocalizing = 1.20 \pm 0.03, and zebra finch non-singing = 1.20 \pm 0.04). Although we did not quantify expression intensity in this study, we also observed high intensity of *FoxP1* in ventral and medial striatum (Figure A2-1). We compared the striatal vocal control nucleus/adjacent striatum ratio among groups statistically. A Kruskal-Wallis test showed no statistical difference among groups (Figure A2-2, $\chi^2=6.74$, d.f=3, $p=0.08$).

FoxP2 and FoxP1 protein expression in budgerigar MMSt and zebra finch Area X

To investigate species differences at protein level, the number of FoxP2 and FoxP1 positive cells in non-vocalizing budgerigars and non-singing zebra finches were compared. To eliminate the effect of possible differences in cell density across regions, the number of FoxP2-positive or FoxP1-positive cells was normalized by dividing by the total number of DAPI-labeled. FoxP2 expression in the MMSt was lower compared to the adjacent striatum whereas a similar level of expression was found between Area X and the adjacent striatum in zebra finches (Figure A2-3). The mean ratio with SEM (striatal vocal control nucleus/adjacent striatum) of the budgerigar non-vocalizing group was 0.70 \pm 0.08, and that for zebra finch non-singing group was 0.92 \pm 0.07. There was a significant difference between the two species (Figure A2-4, Wilcoxon test, $\chi^2=4.08$, d.f.=1 $p=0.04$), with a higher ratio in zebra finches.

FoxP1 protein expression was observed in both budgerigar MMSt and zebra finch Area X, and its expression level was similar to that in the adjacent striatum (Figure A2-3). The mean ratio with SEM for budgerigar non-vocalizing group was 1.01 \pm 0.03, and non-singing zebra finch

group was 1.00 ± 0.04 . No significant difference was found in the ratio (striatal vocal control nucleus/adjacent striatum) of FoxP1 expression between the groups (Figure A2-4, Wilcoxon test, $\chi^2 = 0.08, d.f=1, p=0.77$).

Although quantification was not performed, we observed that FoxP2-labeled cells were usually co-localized with FoxP1-labeled cells (Figure A2-5, co-localized cells indicated with white arrows). While the intensity of FoxP1-labeled cells was uniform throughout the striatum, some variation in the intensity of FoxP2-labeled cells was observed. Strongly labeled FoxP2 cells were found along the ventricular zone in the striatum and the lamina between the striatum and the nidopallium (N), which is directly above the striatum. In contrast, the majority of FoxP2 labeled cells in the MMSt and Area X were weakly labeled (Figure A2- 5).

Discussion

Summary of findings:

In this study, we examined expression patterns of both mRNA and protein of *FoxP2* and *FoxP1* in an adult vocal learner, the budgerigar. We focused on expression patterns in the striatal vocal control nucleus, MMSt, which is a key part of the parrot vocal learning pathway, and examined changes within the MMSt across different vocal states.

We discovered that, regardless of the vocal states (female directed vocalizing, undirected vocalizing and non-vocalizing), *FoxP2* levels are lower in the MMSt relative to levels in the adjacent striatum in budgerigars. Previously, *FoxP2* expression patterns in the songbird striatal vocal control nucleus Area X were found to be driven by the particular singing behavior of adult zebra finches, which are closed-ended vocal learners. In adult zebra finches, when males produce their songs alone, both the mRNA and protein decrease in Area X compared to baseline levels in non-singing birds [8,12,13]. In contrast, when male zebra finches sing to females, the level of *FoxP2* mRNA in Area X remains similar to that in the adjacent striatum, whereas the Area X protein level decreases. In zebra finches, the effect of social context on *FoxP2* mRNA is mediated by social regulation of a *FoxP2*-targeting miRNA [29]. In this experiment, we included a zebra finch non-singing group to provide a direct comparison with budgerigars. We found similar mRNA patterns to a previous study [12]: the expression level of *FoxP2* was similar between Area X and adjacent striatum in non-singing zebra finches. Using immunohistochemistry, we also observed a similar number of FoxP2 labeled cells between these two areas in non-singing zebra finches. Previously, it has been reported that the amount of FoxP2 protein between these two areas is similar in zebra finches under the same behavioral conditions

using Western blot [8]. We cannot compare protein levels directly between the two studies since protein levels were measured in different ways; however the different approaches highlight the same pattern of FoxP2 protein expression in non-singing zebra finches. In contrast, in the budgerigar we found lower levels of FoxP2 protein in MMSt than in adjacent striatum across all groups and this ratio was significantly lower in all budgerigar groups than in the non-singing zebra finches. Taken together, these studies suggest that down-regulation of *FoxP2* is associated with vocal plasticity in both open-ended and closed-ended vocal learning avian models.

On the other hand, we found high mRNA and protein *FoxP1* expression in the striatal vocal control nucleus of both budgerigars and zebra finches (MMSt and Area X) regardless of their vocal states. Using the ratio of striatal vocal nucleus and adjacent striatum, there were no significant differences among groups at either mRNA or protein levels. High level of *FoxP1* was seen in previous studies in songbirds [6,7] and singing behavior did not affect expression level [9]. Therefore, our result strengthens the idea that FoxP1 expression in song nucleus is not vocal driven even in open-ended vocal learners.

We found no relationship between calling rates and levels of expression of either *FoxP2* or *Fox P1*. We focused primarily on contact calls as these are the most commonly produced elements of the budgerigar repertoire. Further investigation of the effect of warble songs on expression of these genes would be worthwhile, though, as they have been shown to affect MMSt expression of the immediate early gene *egr1* [24]. Budgerigars produce warble songs more consistently when they are housed together (E. Hara and T. Wright, pers obs). However, for consistency with previous studies examining *FoxP2* expression, we recorded males either in isolation, or housed separately from females (for the directed group). Further study of the effect of warble song on *FoxP1* and *FoxP2* expression would require modification of this approach.

Role of FoxP2 and FoxP1:

It has been suggested that *FoxP2* down-regulation may play an important role in permitting adult song plasticity in zebra finches. Zebra finches that sang more variable undirected songs showed lower *FoxP2* mRNA expression in Area X compared to adjacent striatal area while levels were similar between these areas when birds were either non-singing or sang less variable female-directed songs [8,12]. Knock-down of *FoxP2* in Area X of juvenile zebra finches via viral-mediated shRNA manipulations prevented animals from copying tutor songs accurately [10,11], which might be due to decreased dendritic spine density in Area X [30]. Furthermore, disrupting *FoxP2* in Area X in adult zebra finches altered song variability, possibly via dopamine receptor dependent modulation in the corticostriatal pathway [11]. In contrast to patterns in zebra finches, we found low levels of *FoxP2* mRNA and protein in the MMSt regardless of the vocal status in adult budgerigars. Such persistent down-regulation is consistent with the fact that budgerigars are capable of modifying their contact calls as adults [21]. Previously it has been reported that *FoxP2* mRNA expression in adult budgerigars is similar between MMSt and the surrounding striatum [6], a result that differs from ours here. This difference may be due to the use of sagittal sections in [6], as the gradual decrement from medial MMSt to lateral MMSt that we observed using coronal sections is not apparent on an individual sagittal section, or it may be due to the shorter behavior sessions before sacrifice used in the previous study. Our results suggest the novel hypothesis that a consistently low level of *FoxP2* expression in MMSt permits the persistent vocal plasticity and open-ended learning observed in adult budgerigars.

The outer region of MMSt is thought to be involved in body movement in various avian models [31]. Humans have the ability to learn movements, such as dancing. Likewise, parrots

have the ability to learn a complex movement by mimicking and performing rhythmic synchronizations like tapping to an audio-visual metronome [32,33]. Therefore, it is possible that the adjacent striatum is involved in other motor learning and *FoxP2* also plays a crucial role in the area. Interestingly, this gradual down-regulation pattern in the striatal area of the budgerigar was also found for the calcium binding protein, calbindin in the budgerigar [34], whereas calbindin is highly expressed in Area X of male zebra finches [35]. Calbindin acts to buffer calcium, which may protect cells from otherwise harmful intracellular levels [36]. The degree of interaction between *FoxP2* and calbindin is unclear. However, both molecules may play critical roles in differentiating open-ended from closed-ended vocal learners, and further investigation is warranted.

Our immunohistochemical results revealed variable intensity levels of staining for *FoxP2* protein across individual cells in the MMSt. Since our immunohistochemistry was performed with fluorescent labeling, staining intensity varied between sections. Therefore, we did not quantify the intensity of labeled neurons in this study. However, most of the labeled neurons within the MMSt appeared to be of low intensity, with high intensity neurons present mainly at the lamina between the striatum and the nidopallium, and also at the ventricular zone. It has been reported that newly born neurons express high intensity *FoxP2* signals in Area X of zebra finch [13]. Therefore, lamellar distribution in budgerigar may represent new neurons that will eventually migrate into MMSt. Moreover, in adult zebra finches singing behavior decreases the number of weakly stained *FoxP2* neurons whereas strongly labeled *FoxP2* neurons were not affected [13]. Budgerigars, however, mainly demonstrated weak staining in the MMSt regardless of their vocal states, which is consistent with ongoing vocal plasticity.

Some literature suggests that *FOXPI* is also involved in human speech [14-19]. In addition, a mutation of this gene is found in some individuals with autism, for which one of the main characteristic is communication and language difficulties [16,37]. *FOXPI* is also involved in organ development, including the heart, lungs, and esophagus [38,39]. In the central nervous system of mice, *FoxPI* plays an important role in the definition of columnar identity of motor neurons in the spinal cords [40], and a recent report showed that it is involved in the development of medium spiny neurons in the striatum [41]. Taken together, these studies suggest that cellular differentiation is a primary function of *FoxPI*. In avian forebrains, high *FoxPI* expression patterns are conserved in the striatum, dorsal and ventral mesopallium [42]. In vocal learning songbirds, *FoxPI* is highly expressed in various vocal control nuclei, including the striatal vocal nucleus, but unlike *FoxP2*, the expression levels do not appear to be driven by age or singing states [9,12]. Therefore, the high expression of *FoxPI* may be crucial for maintaining the organization of vocal nuclei in both open-ended and closed-ended vocal learners.

It is still unclear what upstream factors control *FoxP2* and *FoxPI* expression. However, recent study in rodents showed that when exogenous androgen was administered, both mRNA and protein expression of *FoxP2* and *FoxPI* increased in the striatum, and vocalizations were also altered [43]. Interestingly, androgen receptor expression is high in Area X of zebra finches [44], but low in MMSt in budgerigars [45]. Therefore, it is possible androgens play important role on vocal plasticity, which separate open-ended from closed-ended vocal learners.

Conclusion:

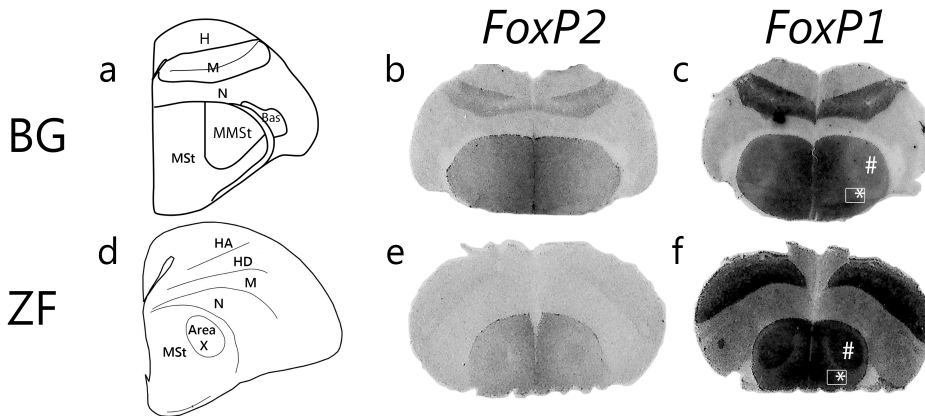
There are some similarities between the development of human language and bird vocal repertoires including babbling-like vocalization at early development, an early critical period of rapid learning, and the importance of auditory feedback [46]. Like humans, budgerigars have the ability to learn vocalizations throughout their lifetime. Consequently, further investigations of molecular mechanisms for vocal learning in this species may offer insight into the maintenance of adult vocal plasticity in humans. In this study, we documented for the first time expression patterns of *FoxP2* and *FoxP1* at mRNA and protein levels in different vocal states in the striatal vocal nucleus of budgerigars. Manipulative studies of gene expression will be necessary to test the mechanism of action of these molecules in adult vocal learning. It has been established that viral manipulations of these molecules are effective in songbirds [10,11], therefore, both overexpression and knock-down of these genes should be feasible using similar approaches in budgerigars. Such experiments in open-ended vocal learners like the budgerigars will offer new insights into the neural and molecular mechanisms of adult vocal learning ability in humans.

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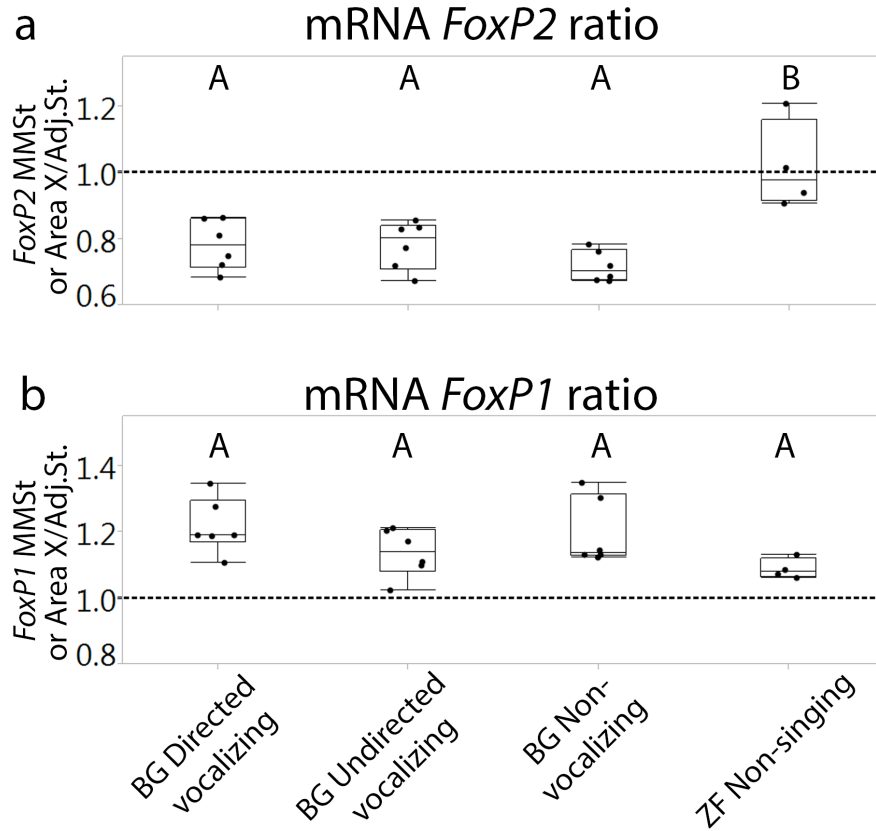
Figures

Figure A2-1: *FoxP2* and *FoxP1* mRNA expressions in budgerigar and zebra finch brain.



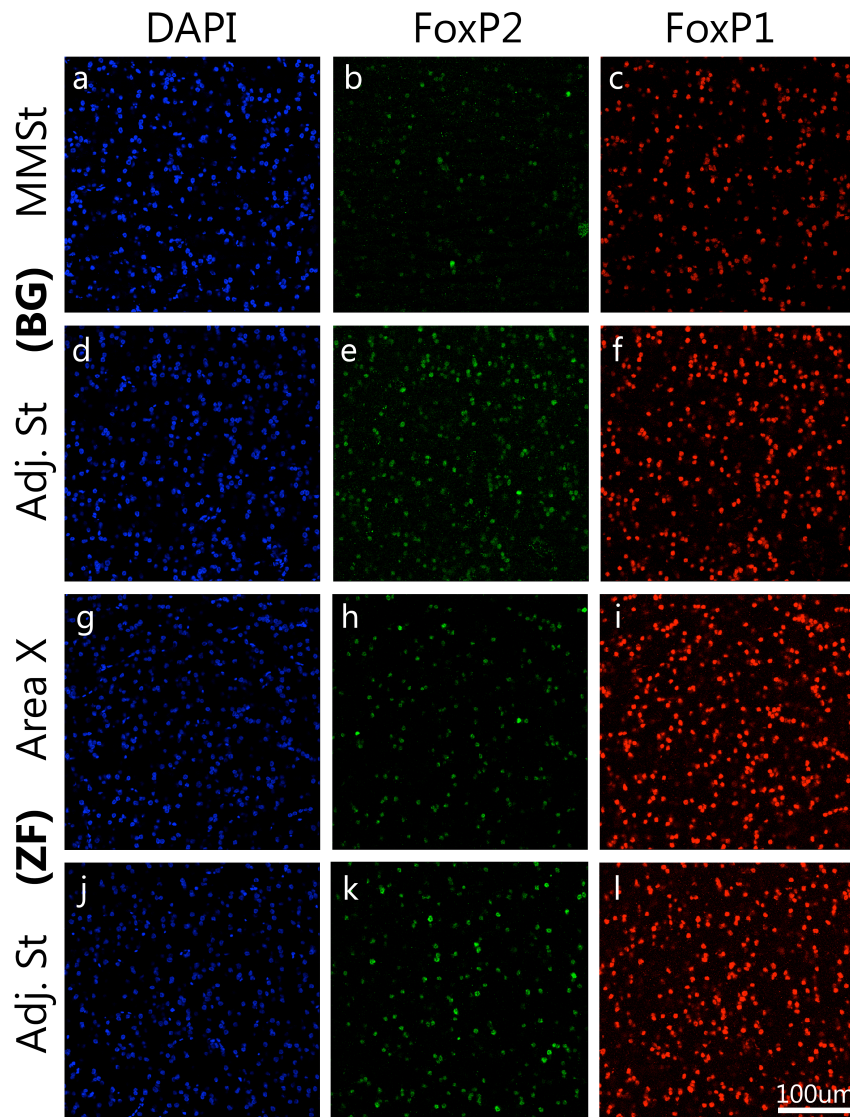
Schematic drawing of brain sections from adult male budgerigars (a) and zebra finch (d). Photomicrographs of brain sections from non-vocalizing adult male budgerigars (BG, top) and non-singing adult male zebra finches (ZF, bottom). Location of striatal vocal nuclei and adjacent areas in schematic sections adopted from the atlas at Reiner et al., 2004 [47]. (b and e) *In situ* signals for *FoxP2*. (c and f) *In situ* signals for *FoxP1*. Boxes indicate the approximate areas of measurement: striatal vocal control nucleus (MMSt for budgerigars and Area X for zebra finches) and adjacent striatum. * indicates the adjacent striatum area where mRNA was quantified, and # indicates that for protein expression. *FoxP2* levels appear lower in the MMSt compared to the adjacent striatum while Area X exhibits similar or slightly higher expression level compared to adjacent area. In contrast, *FoxP1* is highly expressed in the striatal vocal control nucleus in both species. Since zebra finch tissue produced stronger signals, the representative pictures for the two species were taken from different films with different exposure times. Abbreviations: H, Hyperpallium; M, Mesopallium; N, Nidopallium; Bas, Basorostral pallial nucleus; MMSt, Magnocellular nucleus of the medial striatum; St, striatum.

Figure A2-2: *FoxP2* and *FoxP1* mRNA expression ratio in different groups.



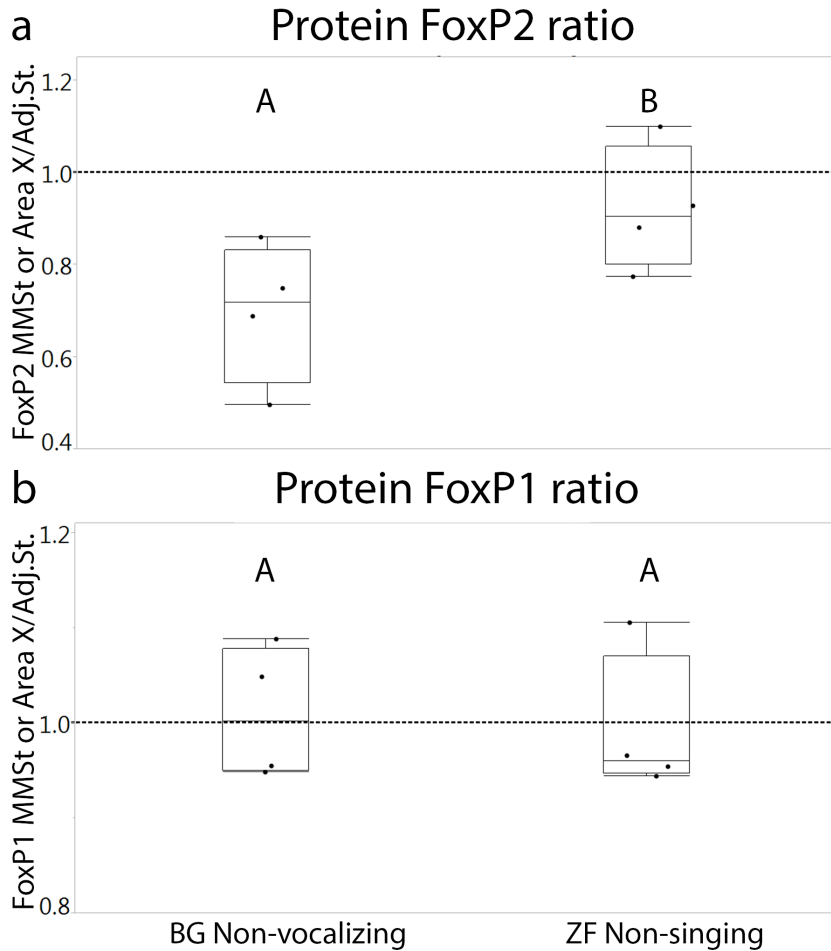
The ratio 1 on the Y-axis indicates the same expression levels in striatal vocal control nucleus and adjacent striatum. (a) There are significant differences between all budgerigar groups and zebra finches for *FoxP2*. (b) The expression ratio of *FoxP1* demonstrates no significant difference among groups. Different letters above the box plots indicate significant differences (p-values in the text). BG=budgerigars, ZF=zebra finches.

Figure A2-3: Immunohistochemical detection of FoxP2 and FoxP1 proteins.



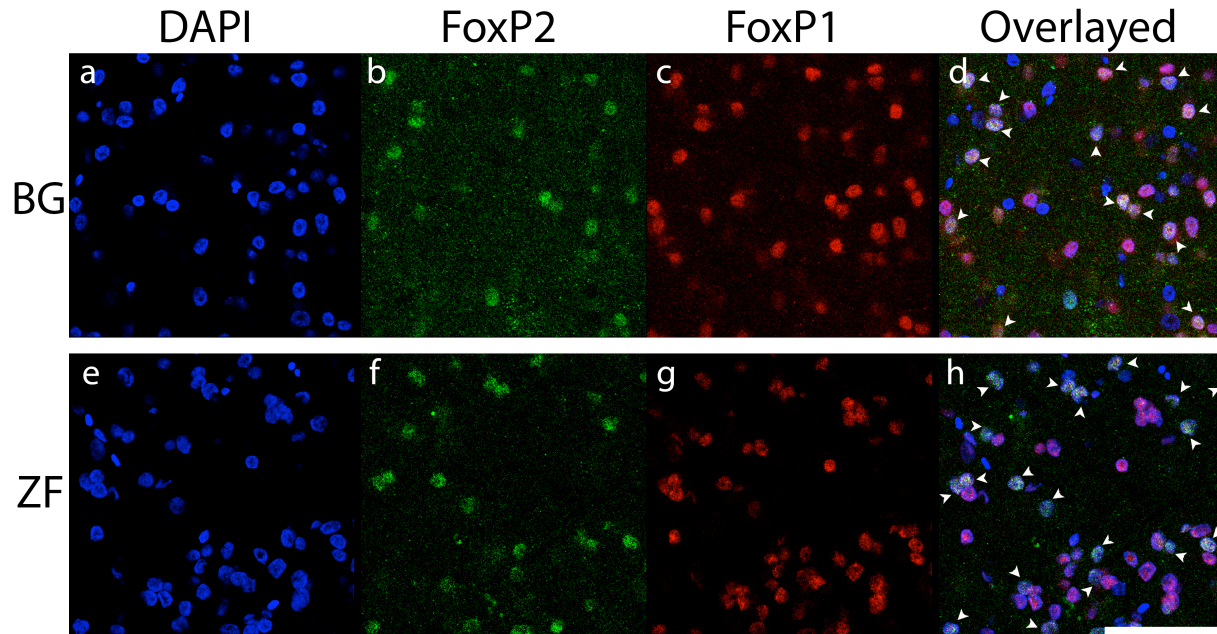
Top two rows are budgerigars (BG: a-f) and bottom two rows are zebra finches (ZF: g-l). DAPI staining exposes all the cells in the area (Blue: a, d, g, j). FoxP2 (Green) reveals a lower expression in the MMSt compared to the adjacent striatum while the expression level is consistent throughout the striatum in zebra finches (b, e, h, k). Red signal indicates FoxP1-positive cells, which demonstrate constant expression levels throughout area and species (c, f, i, l). Scale bar = 100 μ m.

Figure A2-4: FoxP2 and FoxP1 protein expression ratio in non-vocalizing budgerigar and non-singing zebra finch groups.



The value 1 on the Y-axis demonstrates the same level of expression between the striatal vocal nucleus and the adjacent striatum. (a) A significant difference in the FoxP2 expression ratio was found between the two species. (b) There is no difference between budgerigars and zebra finches in FoxP1 levels. Different letters above box plots indicate significant differences (p-values in the text). BG=budgerigars, ZF=zebra finches.

Figure A2-5: High power image of DAPI, FoxP2, and FoxP1 protein signals in striatal vocal control nucleus of budgerigars and zebra finches.



Blue indicates DAPI (a and e), green indicates FoxP2 (b and f) and red indicates FoxP1 labeled cells (c and g). There are more FoxP2 expressing cells in zebra finches' Area X than budgerigars' MMSt. Most of FoxP2 labeled cells are co-localized with FoxP1 as indicated by the white arrows (d and h). Scale bar = 50 μ m.

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