DIFFERENTIAL GENE EXPRESSION IN THE ANTERIOR

FOREBRAIN PATHWAY NUCLEUS AREA X DURING RAPID

VOCAL LEARNING

A Senior Scholars Thesis

by

DUSTIN THAD WHITAKER

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

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Associate Dean for Undergraduate Research: Robert C. Webb

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ABSTRACT

Differential Gene Expression in the Anterior Forebrain Pathway Nucleus Area X During
Rapid Vocal Learning. (April 2010)

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Vocal learning is the complex process by which an organism is able to modify its vocal
output, such as birdsong or human speech, due to experience. The pathways used in the
production and modification of human speech and birdsong have been shown to be quite
similar, and so, the determining the transcriptome changes in songbirds provide a logical
first step to learn more about human speech development. In the current study, trained
Zebra Finches, a passerine songbird, were allowed to progress through only the initial
stage of vocal development, as determined by a pitch increase compared with untrained
isolates. The transcriptomes of the four song nuclei and three auditory forebrain regions
of these two groups were compared using microarray hybridizations, and the results
were confirmed using in situ hybridization. In Area X, part of the anterior forebrain
pathway known to play a role in vocal learning, 149 genes were found to be
differentially regulated, with approximately 85% of these genes decreasing in
expression. Of the differentially expressed genes, some have already been found to play
a role, either directly or indirectly, in learning through previous studies, though most

have still yet to have their properties determined. This study, though important in and of itself, is only the first of many pieces to the large process of vocal learning to be put into place; further work will be able to expand upon work here to fill in gaps in our knowledge of the vocal learning process.

DEDICATION

To those twelve birds sacrificed for the sake of science…

ACKNOWLEDGMENTS

NOMENCLATURE

AFP	Anterior forebrain pathway
BOS	Bird's own song
CMM	Caudal portion of the medial mesopallium
DLM	Magnocellular nucleus of the dorsolateral thalamus
DM	Dorsal medial nucleus of the midbrain
DPH	Days post hatch
L2	Region 2 of Field L
lMAN	Lateral magnocellular nucleus of the anterior nidopallium
NCM	Caudal portion of the neostriatum
nXIIts	Tracheosyringeal subdivision of the twelfth nucleus
PVP	Posterior vocal pathway
RA	Robust nucleus of the arcopallium

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CHAPTER I

INTRODUCTION

Vocal learning has been defined as the ability of animals to modify acoustic and syntactic structure of sounds produced, including imitations and improvisations as a result of experience with others (Konishi, 1969). Vocal learning, unlike auditory learning or the ability to make sound associations has only been documented in a few species. This exclusive group includes three groups of birds; oscine songbirds, parrots, and hummingbirds; and five groups of mammals; humans, bats, cetaceans, and the two recent discoveries of elephants and seals (Reiss et al., 1997; Boughman, 1998; Poole et al., 2005; Sanvito et al., 2007)). While many of these groups have contributed to research, the oscine songbirds, with the other two groups of birds to a minor extent, have been the most well studied of any vocal learners, except humans, since they were first discovered to participate in this type of learning in the 1970s (Nottebohm et al., 1976). Work done has shown that the bird groups, as well as humans, exhibit similarly designed central nervous system circuits for vocal learning and vocal production, despite differences in the design and developmental organization of their peripheral vocal organs, the avian syrinx and the human larynx (Fig. 1 (Jarvis, 2004)). Along with the similar brain structures and pathways, vocal learning itself has many parallels between the avian species and humans including: infant (juvenile) babbling, an early critical

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This thesis follows the style of The Journal of Neuroscience

period for learning and a requirement for auditory input and practice. Examples of isolated or deaf humans and birds the inability to learn proper speech without these four criteria being fulfilled ((Thorpe, 1958; Marler, 1970; Fromkin et al., 1974; Doupe and Kuhl, 1999). As both avian species and humans utilize similar brain and learning mechanisms, research conducted with songbirds may have potentially significant implications for understanding human speech development and vocal motor control.

Figure 1. Comparative auditory and vocal brain regions of birds and humans. Posterior vocal pathways shown in yellow; anterior forebrain pathways shown in red; auditory forebrain regions are shown in light blue. Scale bar ~7 mm. (Figure from Jarvis, 2004)

Structures of the song system

The Zebra Finch (*Taeniopygia guttata*) has been the best studied of all non-human vocal learners. Three brain pathways and regions of interest which have been found in Zebra Finches have been implicated in vocal learning and production, with the other bird groups containing similar, though differently named, structures. The posterior vocal

pathway (PVP) consists of connections projecting from the nucleus HVC (now used as a proper name) to the robust nucleus of the arcopallium (RA) to the dorsal medial nucleus of the midbrain (DM) and tracheosyringeal subdivision of the twelfth nucleus (nXIIts) which ultimately innervates the syrinx (Fig. 1). The anterior forebrain pathway (AFP) is a loop of nuclei which has projections to each other and also input from, and output to, the posterior vocal pathway; this loop consists of the lateral magnocellular nucleus of the anterior nidopallium (lMAN) projecting to Area X to the magnocellular nucleus of the dorsolateral thalamus (DLM) and back to lMAN. lMAN in turn projects to RA, which represents a point of convergence of the PVP and AFP (Foster and Bottjer, 2001). The auditory forebrain regions are: the caudal portion of the neostriatum (NCM), the caudal portion of the medial mesopallium (CMM), and field L (Fig. 1 and 2A; (Striedter, 1994; Vates et al., 1996; Durand et al., 1997; Gahr, 2000; Reiner et al., 2004).

The structures located in the anterior vocal pathway play complementary but opposite roles in the song learning process. Juveniles containing Area X without lMAN produced 'monotonous repetitions of a single note complex;' those containing lMAN without an Area X produced extremely variable song structures (Scharff and Nottebohm, 1991). This demonstrates effectively the competing influence of both Area X and lMAN on song stability. The mechanisms behind these dual interests in song structure are still unknown, in juveniles as well as adults.

ZENK mRNA expression in both NCM and CMM has been shown to positively correlate with the level of similarity between the bird's own song (BOS) and the tutor song, also implicating these areas in song memory (Bolhuis et al., 2001; Terpstra et al., 2004). Moreover, disruption of molecular signaling mechanisms in NCM during the exposure of tutor song blocks song learning (London and Clayton, 2008). The anterior forebrain pathway is thought to regulate both song learning in juveniles and song plasticity in adults (Brainard and Doupe, 2000; Solis and Doupe, 2000; Olveczky et al., 2005; Aronov et al., 2008). Since females do not sing in Zebra Finches, and therefore do not learn song, the nuclei of the AFP are absent, but in species in which both sexes sing, all song nuclei can be found in both sexes (MacDougall-Shackleton and Ball, 1999).

The process of vocal learning

In Zebra Finches, only the males are capable of vocal learning and, once mature, adults produce a single, highly stereotyped, song. This process occurs in two overlapping stages: the sensory and sensorimotor phases (Fig 2B). In the sensory phase, juveniles acquire a template of their tutor's song; this phase begins at approximately 25 days post hatch (dph) and spans until 60 dph (Roper and Zann, 2006). Overlapping with this initial phase is the sensorimotor phase in which the juvenile birds begin to practice singing their own song and gradually modify it to match that of their tutor. Song structure becomes more crystallized and unable to be modified to a great extent after 90 dph, when the bird becomes an adult. The timelines for this two-stage process are found in Figure 2B. Although the process of modification of vocal output of the juvenile's
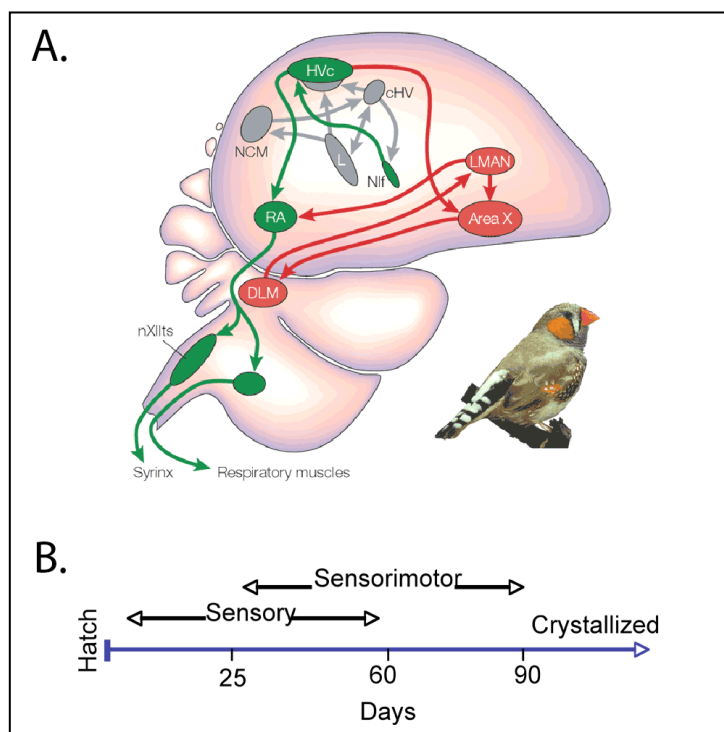
Figure 2. The auditory and vocal systems. **A)** The anterior forebrain pathway and its connections is labeled in red, the posterior vocal pathway is in green, and the forebrain auditory areas are in grey. cHV, above, is the outdated nomenclature for CMM. To the right is a picture of an adult male Zebra Finch. (Figure from Brainard and Doupe, 2000) **B)** Timeline of critical period in Zebra Finches (Modified from Brainard and Doupe, 2002)

song towards that of its tutor extends for months, changes in the temporal and spectral structure of song have been seen after only a single training session of approximately 75 seconds of exposure of tutor song. This short amount of exposure has been shown to produce induce song imitation in juvenile males as tested at 90 dph (Figs. 3 & 4). Thus, a single session of operant training can time lock the beginning of neural processes occurring in the juvenile bird's brain during his march through vocal learning. Before this study, changes in gene expression of the songbird vocal control nuclei during periods of vocal learning had not been properly explored; instead, most studies have

shown only the beginning and end results, with a lack of information of what is occurring throughout the process.
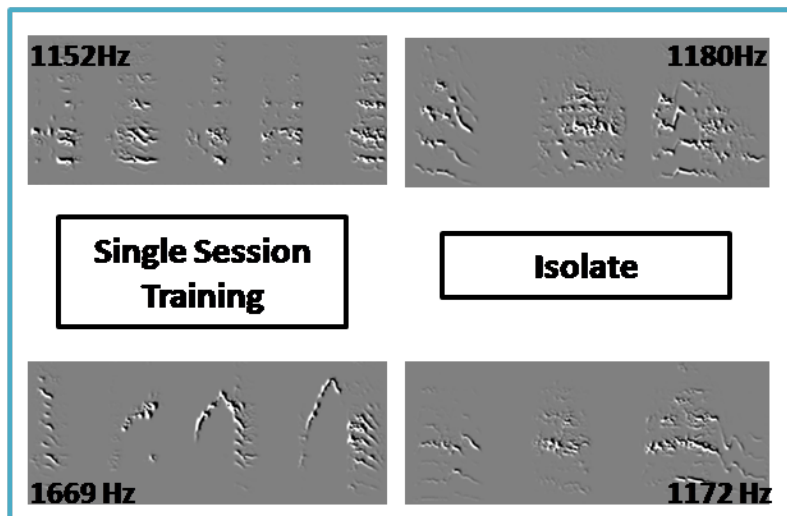
Figure 3. Spectral changes after single session. The spectral diagrams show that the song output becomes more organized, as well as increases in pitch, after only a single training session. The songs of both birds are seen here after a lapse of 8 hours.
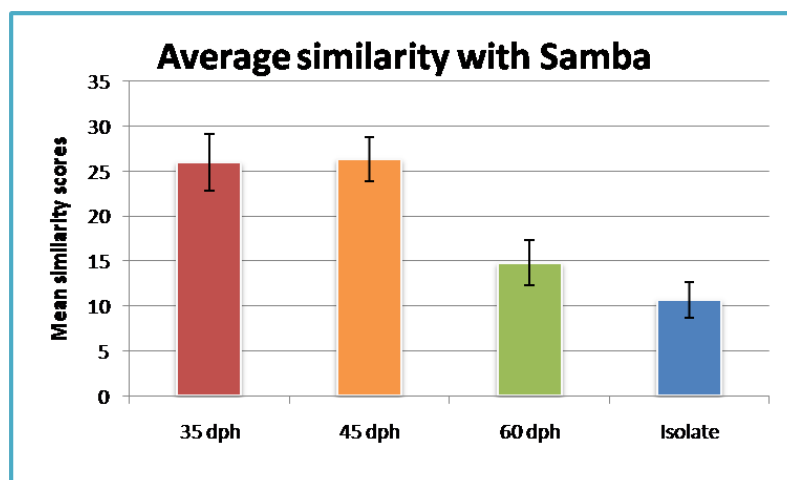
Figure 4. Single training session similarity comparison. Birds were trained using the same protocol as the current study (see Methods); the similarity of imitation to that of the tutor song (Samba) is seen to be highest in the early sensitive period for vocal learning (35/45 dph). This figure shows that a single session significantly increases song imitation compared to that of an isolate.

The genomics era

Since the beginning of the genomics era, high-throughput methods, such as the microarray, have been developed to generate large sets of data and interrogate biological processes in a quicker and more efficient way. Genomes have been sequenced in many species, with more likely completed in the near future, and genes skipped over in the past are finally finding their rightful place in the regulation of cellular processes. The Zebra Finch has not been left out of all of these genomic experiments as its genome has just been fully sequenced (Warren et al., 2010) and multiple other genomic experiments have recently brought forth new data that has previously been unknown. Microarrays have been used recently to obtain differential gene expression between multiple groupings of birds to find gene products that might be regulated as a consequence of adult singing and/or song perception ((Wada et al., 2006; London et al., 2009). A recent description of the Zebra Finch genome (Warren et al., 2010) reveals that 17,475 protein-coding genes are represented in the songbird genome, of which approximately 57% are expressed in the brain. A significant amount of these neurally-expressed genes (~900) are differentially regulated between the 50 dph juvenile brain, in the sensitive stage for song learning, and adult (2.5 years) male brains. Though changes between these two distinct stages might show differences based on experience or age, it lacks a way to properly represent genes that might only be activated, or deactivated, during transitory stages of vocal learning. This study puts the song nuclei of the Zebra Finch through a rigorous analysis in the hope of providing some insight into the genomic changes occurring during the first days of the vocal learning process.

CHAPTER II

METHODS

Animals

The current study used juvenile male Zebra Finches (42 – 48 dph; n=6/group), which were raised in breeding colonies in our laboratory. These males were isolated from their fathers prior to fledging at 7 – 10 dph and were raised by their mothers until reaching an age of independence, at approximately 35 dph. The juveniles were relocated again into sound isolation boxes at this stage. All animal procedures were carried out under guidelines established by the Texas A&M Institutional Animal Care and Use Committee, under approved Animal Use Protocols 2006-21 and 2009-35.

Operant training paradigm

The isolation boxes contained two Zebra Finch models, a camera with microphone, and a speaker; a key string was attached to the female model while a speaker was located behind the male model on the opposite end of the cage (Fig. 5). Continuous recordings were taken during the entire training program, and from these recordings, the subject's subsong, its auditory output prior to receiving any training, and its post-training song development were analyzed using Sound Analysis Pro software (Tchernichovski et al., 2004). Each bird was allowed to pull at the string to trigger song playback, with a maximum of forty playbacks given per day (2 sessions/day; 20 playbacks/session), as per earlier experiments (Tchernichovski et al., 2001). The number of songs was limited

due to the fact that too many playbacks in a given time have been found to be detrimental to song learning (Tchernichovski et al., 1999).

Once the song of the trained birds was found to have a definitive increase in song pitch, relative to its untutored baseline pitch, the birds were allowed to have approximately 30 minutes of singing before sacrifice. Thirty minutes of song has been found to maximize the expression levels for some activity regulated genes (Jarvis and Nottebohm, 1997). Birds were sacrificed by decapitation, and the brains were fast frozen in -40°C isopentane and mounted in optimal cutting temperature (OCT) gel. Untrained birds were isolated in the sound chambers for an approximately equal amount of time and also sacrificed after a singing bout of 30 minutes. The brains were sectioned using a Leica CM1850 cryostat at 10 and 12 μm for the in situ hybridizations and microarray hybridizations, respectively.

Figure 5. Operant training paradigm setup. The birds were isolated in an acoustic chamber. Audio/video recordings were taken throughout the entire session using the camera (left) and microphone (not shown). After pulling the string key attached to the female model (left), song playback would come through from the speaker (right) behind the male model.

Microarray hybridizations

Sections from the left hemispheres of the birds were dehydrated in washes of 70, 95, and 100% ethanol and fixed with xylenes in preparation for laser capture microscopy. Once fixed, four song nuclei (HVC, RA, Area X, lMAN) and three regions of the auditory forebrain (NCM, CMM, L2) were isolated and the mRNA was extracted using the PicoPure® RNA Isolation kit (Molecular Devices). Using the µMACS™ SuperAmp™ kit (Miltenyi Biotec), the mRNA was reverse transcribed and amplified to yield cDNA; this same kit was utilized to couple the Cy-3 fluorophore with the cDNA products. Microarrays were hybridized using the Gene Expression Hybridization Kit (Agilent).

The arrays were scanned at a 5μm resolution on a GenePix 4000B microarray scanner using the Agilent Feature Extraction Software v9.5.1.

Microarray analysis

Following the hybridization and scanning procedures for the microarrays, a number of control steps were taken in order in ensure that the microarray results were of high quality. Negative controls on the microarrays are measured; probes that were not significantly higher than that of the negative controls (three standard deviations) were filtered. If almost no probe on an array reached this criterion, the entire array was rejected. An array was also discarded from the analysis if the probe value for that array showed little relationship to the median probe value for all other arrays (Fig. 6). Normalization of the microarrays was done using two assumptions. The first is that most genes, greater than fifty percent, do not change between the two groups and so are normalized to one another. The variance of the expression levels between arrays should be consistent using the mean of the probe increases across arrays. If many of the probes match the same gene, and the expression level across all experiments is highly correlated, the individual probe values are merged to get a single median value for the gene.
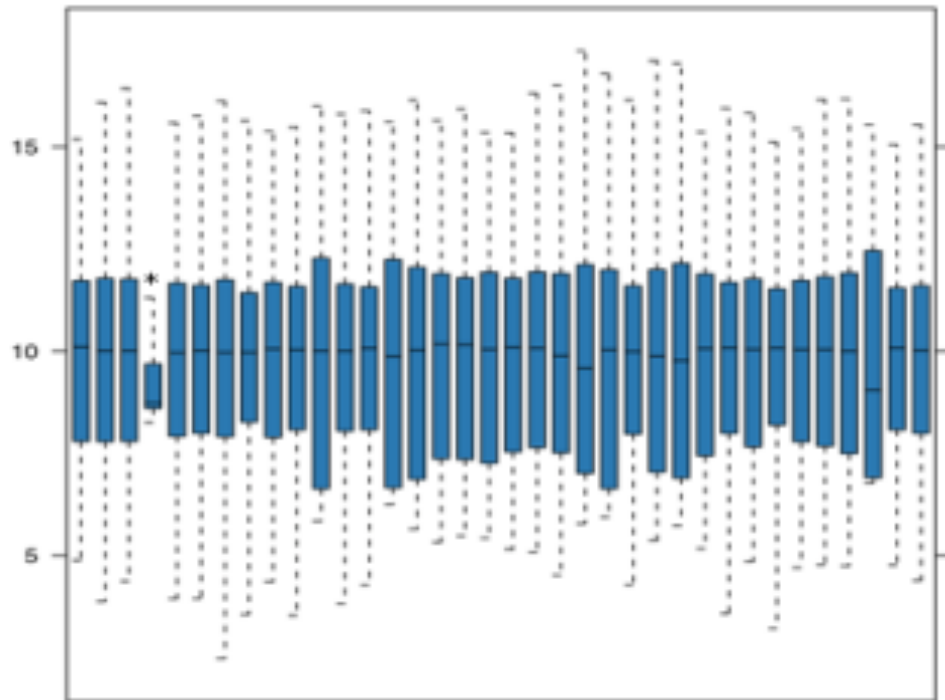
Figure 6. Microarray boxplot analysis. Shown are the M-values for 36 microarrays; arrays must all be similar in expression values. One array (*) shown had to be physically removed from any analysis due to its lack of consistency with the other arrays.

Differential gene expression between the two groups was tested using a linear model for individual brain regions. In this case, a t-test was used to determine if the gene is significantly different between the two groups. A problem using a simple t-test is the microarray's potential for type I errors, a problem when analyzing microarray data (Benjamini and Yekutieli, 2005); this problem is corrected by applying a false discovery rate (FDR) p-value to the samples. All samples found to be significant in the present study met the adjusted FDR p-value of 0.25.

In situ hybridizations

Every tenth section was fixed using 4% paraformaldehyde and the hybridizations were performed using the protocol described by Mello and Clayton, with the following adjustments: [^{33}P] was used in place of [^{35}S]; 120 µl of hybridization solution was used and covered using the adjacent slide; the slides were incubated in mineral oil overnight; slides were exposed to phosphoimager screens (X) in place of the X-ray films (Mello and Clayton, 1995) . Slides were exposed to the screens for a period of 18 – 24 hours and scanned using FujiFilm BAS-5000 Phosphoimager. Adjacent slides were stained using cresyl violet to identify brain regions.

In situ hybridizations were performed as an independent confirmation of the microarray results on a subset of the genes. Genes tested represented genes from different nuclei in the song system in order to confirm each set of microarrays. Analysis of the images was done using the ImageJ analysis program provided by the National Institutes of Health (Sheffield, 2007). Using this software, a ratio of the mean pixel intensities of the region of interest over that of the region's background were taken for each gene in each region. A simple two-tailed t-test was used to determine significance; since only one gene is tested at a time, an adjusted FDR p-value is not necessary for this analysis.

CHAPTER III

RESULTS

Of the 44,000 features on the microarray, corresponding to approximately 17,000 genes; 149 were seen to change in Area X between birds undergoing rapid vocal change (pitch increase, n=6) and birds singing untutored song. 23 (15.4%) were seen to be up regulated while 126 (84.6%) decreased in expression. In the recently sequenced genome of the Zebra Finch, approximately 40% of transcripts found in the auditory forebrain of unstimulated birds were non-coding or are derived from intronic loci (Warren et al., 2010); our results are consistent with these findings in that many of the genes (~17.5% predicted) which decrease in expression in the days after operant song training are non-coding.

Table 1 lists a selection of the genes which have been found to be differentially regulated during this first stage of the vocal learning process; the genes in this list also provide a starting point for genes which will be further tested using in situ hybridization as a complement to the microarray results.

Table 1. Differentially expressed genes.

Clone ID	Gene Name	Area X (+/-)
0063P0022D04	CNTNAP4	+
0203P0043C05	JAG2	+
0205P0012C03	SAP18	+
0206P0011N18	RFNG (DCXR)	-
0202P0004P12	A2BP1 (Fox-1)	-
0203P0011I23	CRTC1	-

The above listed genes were selected highlight some processes in which might be important in vocal production and learning. Four genes in Area X (JAG2, RFNG, SAP18, and CRTC1) are all genes encoding proteins that play a role in the Notch pathway, which has been seen to influence learning (Costa et al., 2003).

Even if these studies seem to be fairly unrelated, the basis for learning and memory consolidation could require similar mechanisms of the Notch pathway.

Another differentially regulated gene that immediately becomes apparent is CNTNAP4, a paralog of the CNTNAP2 gene. The CNTNAP2 gene has been implicated in autism as well as processes of vocal learning and production (Arking et al., 2008; Vernes et al., 2008). CNTNAP2 has also been shown to be negatively regulated by the FoxP2 protein (Vernes et al., 2008), which has been implicated in speech dyspraxia and autism (see discussion). With the close similarity between the two genes, CNTNAP4 might also be a negatively regulated target of FoxP2 during vocal production and have some direct parallels with CNTNAP2.

A2BP1, also known as Fox-1, is also a regulated gene that stands out. This gene is able to autoregulate itself, and the protein product is able to modulate alternative splicing of many downstream targets by inhibiting alternative splicing patterns ((Underwood et al., 2005; Lee et al., 2009; Damianov and Black, 2010). A2BP1's recognition sequence is highly conserved but widely distributed, with thousands of potential targets ((Zhang et al., 2008; Kuroyanagi, 2009). The possible involvement of A2BP1 in regulating whole-

scale alterations to the ratio of non-coding and coding RNAs in the unstimulated and

stimulated brain, respectively, is considered further in the discussion.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

Discussion

Vocal learning, or song learning in songbirds, is a highly complex process that is the result of a combination of sensory and motor processes working in tandem to produce dramatic changes in vocal output, i.e. song. Changes in the transcriptional products during the vocal learning process have not been fully addressed to date, though this study begins to shine some light upon the initial stage of this complex process. Surprisingly, many of the genes found to be differentially regulated in the current study are non-coding and have decreased expression levels post-training. More work will need to be performed to determine Area X's activity levels during the vocal learning process and how this relates to the transcriptional changes we observe. Does the net decrease in transcription in this nucleus at the start of the song imitation process reflect a decreased overall activity in this nucleus? This would suggest that Area X's function of song stability, or its function of inhibiting lMAN's variability function, is decreased during the vocal learning process, perhaps facilitating the birds production of rapid vocal changes at the syllable level (for example, pitch increase). Alternatively, the transition from a higher proportion of non-coding RNAs to a transcriptome with a greater bias towards functional coding RNAs might indicate an increase in Area X neural control. This would tend to inhibit lMAN function and song variability, and perhaps might contribute to the increased temporal stereotypy of syllable delivery (e.g. repetitive

delivery of one or a few syllables) that also characterizes an early phase of vocal learning. Distinguishing between these two possible mechanisms requires further investigation.

Unexpectedly, we also found (not presented here) that relatively few genes that are expressed differently after training in the nucleus HVC. At first thought, this seems to be counter-intuitive during this period of rapid changes to song characteristics due to the fact that the posterior vocal pathway provides the coding signaling for song production. However, the fact that few genes are seen to change in HVC relative across the two groups of birds, seems to make sense when viewed from the perspective that HVC is more tonic, providing essentially a timing code for song production (Long and Fee, 2008) while the nuclei in the anterior forebrain pathway have been shown to produce changes in song structure. Nucleus RA potentially would show greater changes in gene expression levels because of its direct contribution to the spectral properties of song output compared to HVC, which only provides the overall temporal code of the song, and the large pitch changes seen after training (Shank and Margoliash, 2009). It would seem that lMAN would also be producing large amounts of change during this period because of the nucleus' activity in adding variability to song output, though this hypothesis has yet to be tested. Once the transcriptional product changes have been found for lMAN, the relationship between the two main AFP nuclei engaged during song learning will begin to become clearer. As of now, it seems uncertain whether Area X is providing a drive towards stability with lMAN's activity staying constant or if Area

X's activity is fairly constant and lMAN becomes more active in order to overcome the stability effects of Area X, or if the two work in tandem.

CNTNAP4 is one of the many genes down regulated. Many studies have found an inverse relationship between a paralog of this gene, CNTNAP2, and FoxP2. Knockdown of the FoxP2 gene in Area X of the young finches has been found to lead to incomplete or inaccurate vocal imitation (Haesler et al., 2007); levels of this gene were also shown to be significantly lower during adult undirected singing in comparison with directed (to a female) singing (Teramitsu and White, 2006), leading one to think that lower levels of FoxP2 present allows for greater variability during song production. If FoxP2's relationship with CNTNAP4 is similar to that of CNTNAP2 (BLAST gives an e-value of $2*10^{-76}$ between the two mRNAs), it would seem that song variability might also be regulated by FoxP2 through CNTNAP4. Previous studies show the relationship between FoxP2 and many speech and language disorders ((Fisher et al., 2001; Lai et al., 2001; Enard et al., 2002; Vargha-Khadem et al., 2005) CNTNAP4 may provide another mechanism by which FoxP2 is able to affect song imitation and production.

Not only is the CNTNAP4 and FoxP2 relationship one that will be tested, the contribution of other differentially expressed genes to the vocal learning process will also be determined in a later study. A2BP1 is a case in point; this gene has been shown to be important in the regulation of alternative splicing and may be importation in the regulating non-coding RNA population. Another reason for delving deeper into the

function of this particular gene is that Martin et al. (2007) and Morrow et al. (2008) have implicated it as a candidate gene of autism spectrum disorders, a major class of disorders involving deficits in social communication, speech and language (Martin et al., 2007; Morrow et al., 2008). A2BP1 is a negative regulator of splicing (Underwood et al., 2005). It is also decreased in expression after training in the songbirds. If mutations in this gene are able to produce the effects of autism, the mechanism by which this is accomplished could be by continual repression of needed downstream products.

Conclusions

Though the current study has begun to determine Area X's role in song learning, the role of the other song nuclei, in conjunction, will need to be determined in order to obtain the whole picture. This work has already begun; once completed, extensive work will need to be done to determine not only how each gene influences song learning but also how whole networks of genes interact with one another for this common goal.

Our current study will determine the many genetic changes occurring during this first part of the vocal learning process; though it is only one small piece of the puzzle. Later work will need to progress through later stages of song development in order for the entire process to be seen. Although these studies will be able to show more accurately how vocal development progresses in the isolated individual, song learning in the wild and human vocal learning are not processes done by the individual in isolation but are highly influenced by social interactions; determining social influences will be another

large step in bridging the gap in our understanding of these complex developmental

learning processes.

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