THE EXPRESSION OF AUTISM GENES MECP2 AND FOXG1 IN THE ZEBRA FINCH BRAIN DURING VOCAL DEVELOPMENT

AN HONORS THESIS

SUBMITTED ON THE 5 DAY OF MAY, 2023

TO THE DEPARTMENT OF NEUROSCIENCE

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

OF THE HONORS PROGRAM

OF NEWCOMB-TULANE COLLEGE

TULANE UNIVERSITY

FOR THE DEGREE OF

BACHELOR OF SCIENCE

WITH HONORS IN NEUROSCIENCE

BY

Sarah Frances

APPROVED:

Dr. Elizabeth Fucich
Director of Thesis

Dr. XiaoChing Li
Co-Thesis Director

Dr. Julie Alvarez
Third Reader
Sarah Frances. The expression of autism genes MeCP2 and FOXG1 in the zebra finch brain during vocal development.

(Dr. XiaoChing Li, LSU Neuroscience Center of Excellence)

Abstract

Rett syndrome (RTT) is an X-linked neurodevelopmental disorder classified as an autism spectrum disorder. It typically affects young females and causes progressive impairment and loss of motor skills and language. The zebra finch bird is an optimal research model as its song learning process mimics language development in children. Similar to speech in humans, zebra finch song learning happens during a critical developmental period. The song learning circuitry of the zebra finch includes Area X, a basal ganglia nucleus within the anterior forebrain pathway (AFP). Studying vocal learning in the zebra finch may provide insight into human neurodevelopmental disorders.

It is known that mutations in the methyl-CpG-binding protein 2 (MeCP2) gene cause RTT. Although little is known about the relationship between the forkhead box protein G1 (FOXG1) gene and the MeCP2 gene, the FOXG1 gene is thought to be involved in the song learning circuitry. FOXG1 gene mutations can cause FOXG1 syndrome, which overlaps with RTT symptoms. Children with FOXG1 syndrome have severe physical and cognitive disabilities such as intractable seizures, movement disorders, cortical vision impairment, and language difficulties. We investigated the expression patterns of the MeCP2 and FOXG1 genes in the song control nuclei as well as during vocal development in singing versus non-singing birds. Preliminary research determined that the MeCP2 and FOXG1 genes are likely developmentally regulated across the main song nuclei. Also, we found that singing likely acts as a regulatory behavior for gene expression for both the MeCP2 and FOXG1 genes.
Acknowledgements

I am extremely grateful for the mentorship I received from my principal investigator, Dr. XiaoChing Li and thesis director, Dr. Elizabeth Fucich. Dr. Li has been a mentor in the lab since last year when I began an internship with LSU Health Sciences and continues to be a source of support in and out of the lab. Her expertise in the field and willingness to explain concepts to me have deepened my interest in neuroscience. I would like to thank Dr. Fucich for supporting my neuroscience education at Tulane through the Cellular Neuroscience and Experimental Design in Neuroscience classes and lab. The latter taught me important skills related to ethical research conduct, organizing information concisely, and developing confidence in delivering scientific content. I would also like to thank Dr. Julie Alvarez who served as my third reader and provided valuable guidance and feedback throughout the thesis writing process. I would like to thank Hannah Jarrell for training me last summer and acting as a constant resource guiding me through my independent thesis project. My thesis work could not have been possible without the encouragement of my friends and family, along with Bacilio Benelalija and Júlia Romagnoli, two undergraduate students in the lab. I would like to recognize the Newcomb Tulane College Center for Academic Enrichment for supporting me last summer through a summer research grant. Lastly, I am extremely grateful for the LSU Neuroscience Center of Excellence and Dr. Nicholas Bazan for giving me the opportunity to work as a summer intern and develop a better understanding of evolving research methods in neuroscience.
# TABLE OF CONTENTS

Title page .................................................................i

Abstract ........................................................................ii

Acknowledgements ....................................................... iii

Table of Contents .......................................................... iv

List of Figures .................................................................. v

1. Introduction ...................................................................1

   1.1 The zebra finch songbird model ...........................................1

   1.2 Development and Cellular Change .....................................3

   1.3 The MeCP2 gene and Rett syndrome .................................4

   1.4 The FOXG1 gene and FOXG1 syndrome ..........................5

2. Hypothesis .....................................................................6

3. Materials and Methods ..................................................6

   3.1 Animals ....................................................................6

   3.2 Perfused tissue and brain slice preparation .......................8

   3.3 Immunohistochemistry ................................................9

   3.4 Singing versus Non-Singing Birds .................................11

   3.5 Gene Expression – Neuron Specificity ............................11

   3.6 Quantification ..........................................................12

4. Results ..........................................................................12

5. Discussion ......................................................................24

6. Limitations .....................................................................26

7. References ......................................................................29
LIST OF FIGURES

Figure 1: The song learning circuitry of the zebra finch songbird

Figure 2: The critical age points in zebra finch bird song development

Figure 3: Stepwise experimental procedure

Figure 4: MeCP2 gene expression in Area X of juvenile and adult bird brains

Figure 5: 40x view of MeCP2 gene expression in Area X of juvenile and adult bird brains

Figure 6: FOXG1 expression in juvenile and adult bird brains

Figure 7: 40x view of FOXG1 expression in Area X of juvenile and adult bird brains

Figure 8: Signal intensity quantification for FOXG1 gene expression in Area X

Figure 9: FOXG1 expression in the LMAN of a juvenile bird brain

Figure 10: FOXG1 expression in the HVC of juvenile and adult birds

Figure 11: 40x view of FOXG1 expression in the HVC of juvenile and adult birds

Figure 12: MeCP2 gene expression in a singing vs. non-singing bird

Figure 13: Closer look at the MeCP2 expression in singing (Bird ID: light blue 42) vs. non-singing bird (Bird ID: violet48)

Figure 14: Signal intensity quantification for MeCP2 gene expression in Area X

Figure 15: MeCP2 gene expression in a singing vs. non-singing bird

Figure 16: FOXG1 gene expression in Area X of a singing vs. non-singing bird
1. Introduction

1.1 The Zebra Finch Songbird Model

The song circuitry of the zebra finch brain includes crucial song nuclei that forge connections between sensory and motor learning. The system comprises of two interconnected pathways: the direct vocal-motor pathway (DMP) and the anterior forebrain pathway (AFP). The DMP is necessary for song production whereas the AFP, also known as the basal ganglia-thalamocortical circuit, enables song learning and vocal plasticity (Chen et al., 2014). Key nuclei include Area X, the high vocal center (HVC), the robust nucleus of the arcopallium (RA), and the lateral magnocellular nucleus of the anterior nidopallium (LMAN) (Figure 1). Axons from both the HVC and LMAN contribute excitatory input onto the RA interneurons (Spiro et al., 1999).

In humans, a similar region to Area X is the basal ganglia and vocal learning in songbirds can provide a better understanding of autism spectrum disorders in humans. Damage to Area X in juvenile songbirds inhibits song crystallization as this area is the receptor of dopaminergic innervation originating from the midbrain ventral tegmental area (VTA). Area X also plays a role in VTA dopaminergic neuron activity following auditory events (Shi et al., 2018).

Figure 1. The song learning circuitry of the zebra finch songbird (Fee & Scharff, 2010)
Although the FOXG1 gene has previously been studied in mammals in various brain regions, it has not been studied in birds, and we are targeting the gene expression in specific basal ganglia nuclei. Using the bird model for the first time to explore the FOXG1 expression, the data will be important in guiding future directions related to translational studies about human neurodevelopmental disorders. If the data are significant, it will be possible to explore further questions pertaining to Rett syndrome and other autism spectrum disorders.

The zebra finch birds are “closed-ended” vocal learners; therefore, beyond the critical period as adult birds, there is limited ability to change their vocalizations (Chakraborty et al., 2017). The juvenile zebra finches are known as pupils and start by hearing and memorizing an adult tutor’s song. At around 30 days after hatching, these pupils start vocalizing and do so with highly variable songs (Shi et al., 2018). Through sessions of auditory feedback, the juvenile bird learns a song resembling the tutor's.

As seen through Figure 2, within the critical time points, two types of learning occur. Sensory learning occurs from before 30 days to 60 days, and motor learning takes place from approximately 30 days to 90 days (Gobes et al., 2019). Given that the critical learning period ends at 60 days, analyzing gene expression before and after the time point can provide insight into developmental regulation, which is important for RTT, involving sensory-motor and language defects. After the critical period, the length of each syllable or slight pitch changes can occur. By the time the birds have reached 100 days, they have already learned their song and will maintain it throughout their lives.
1.2. Development and Cellular Change

During the song learning period, the cell populations and projection of the song nuclei undergo changes (Olson et al., 2015). Past studies have shown a heightened volume of neuronal portions of the brain and diminishing cell population. Additionally, new neurons can be formed in areas such as Area X and the HVC (Bottjer, 2004). Extension of the song learning period is seen through neuronal death and replacement.

The ability to perform neuronal turnover demonstrates neural plasticity (Larson et al., 2019). Decreases in neurogenesis can be linked with stereotypy in the bird song (Pytte et al., 2007). In humans, autistic children experiencing stereotypy exhibit restricted repetitive behaviors (RRB), shown through fixed, non-functional, and purposeless movements (Ghanizadeh, 2010). Current research is necessary to understand the expression of the FOXG1 gene in the song nuclei and apply it to the human brain, especially with autism spectrum disorders.
1.3 The MeCP2 Gene and Rett Syndrome

Past studies have pointed to MeCP2 mutations as a cause of RTT. Kyle et al. (2018) established that these mutations represent about 95 percent of typical RTT cases. Currently, MeCP2 mutant rodents are a widely used model to study RTT to observe these missense, frameshift, and nonsense mutations. The primary function of the MeCP2 gene is to bind the methyl-Cytosine of DNA and regulate gene transcription. It is typically noted as a transcriptional repressor. This process often involves interactions with transcription factors. For example, Zhou et al. (2022) recently identified the transcription factor 20 (TCF20) complex, a protein interacting with MeCP2 at the chromatin level, and found that RTT-causing mutations in the MeCP2 gene disrupt the interaction. The MeCP2 nuclear protein is largely expressed in central nervous system neurons; however, peripheral neurons also contribute to RTT phenotype (Gadalla et al., 2011).

RTT typically affects females as they possess two X chromosomes. Mutations are not epigenetic; however, they cause epigenetic consequences as the MeCP2 gene can read epigenetic signals (Wood et al., 2013). Most mutations within humans happen de novo, meaning spontaneous and non-inherited, within paternal germline cells, which are transmitted to females but not males. In certain instances, these de novo mutations also can be passed down from mothers or inherited from mothers with mild cognitive impairment or show no symptoms caused by skewed X-inactivation promoting wild-type allele expression (Good et al., 2021). Understanding MeCP2 gene expression in the zebra finch songbird can guide therapeutic advances relieving the clinical consequences of gene mutations and guide research with the FOXG1 gene given the overlap in symptoms. Although the MeCP2 gene has been studied in zebra finch songbirds, the interactions of the MeCP2 and FOXG1 genes as well as potential co-regulation has not yet been explored in the zebra finch songbird.
1.4 The FOXG1 Gene and FOXG1 syndrome

Dr. Alessandra Renieri discovered the FOXG1 gene in 1995 in Siena and FOXG1 syndrome subsequently was determined to be a sister to RTT rather than falling under the same RTT umbrella (Ariani et al., 2008). This gene is an early transcription factor giving rise to the ventral telencephalon, the location where the cerebrum develops prenatally. It is crucial in ensuring proper cerebrum development as this part of the brain controls the central nervous system (Younger et al., 2022).

With the FOXG1 gene, a study found that patients with FOXG1 mutations, such as duplications, deletions, frameshifts, and points mutations, were linked with having congenital Rett syndrome (Wong et al., 2019). The disorder is also known as FOXG1-related encephalopathy. The mutations have similar clinical signs to the MeCP2 mutations, with symptoms ranging from severe cognitive disabilities to language impairment that can severely affect individuals' daily functioning. Additional developmental symptoms include limited purposeful use of hands and most children cannot sit or walk without assistance (Younger et al., 2022). Individuals with FOXG1 syndrome heavily depend on others for everyday help, and this feature increases the need to understand how gene expression and mutations connect to clinical features of the disorder.

Although parents can be carriers for FOXG1 syndrome, most cases tend to be non-inherited with no familial link. Current treatments include medications, nutritional support, and physical and speech therapy to achieve a manageable quality of life (Younger et al., 2022). Given that there are associated conditions such as sensory processing disorder (SPD), cortical visual impairment, and movement disorders, insight into the FOXG1 gene can increase understanding of related disorders.
Current animal research with the FOXG1 gene can fill the gaps in what is known about human RTT disorders and FOXG1 syndrome. By understanding gene expression patterns, therapeutic advances can be applied to humans, and a link can be established between neuropathologies and clinical symptoms. By tapping into FOXG1 syndrome's similarity to other disorders, researchers can collaborate to improve the lives of families affected by FOXG1 syndrome.

2. Hypothesis

It is hypothesized that the expression of the FOXG1 gene will be developmentally regulated and its expression will be heightened in the song nuclei regions. We seek to examine how the gene expression differs throughout the zebra finch songbird neurodevelopment and are predicting that there will be greater expression during the critical song-learning period. We are also hypothesizing that singing will enhance the MeCP2 gene expression in the song nuclei. Similarly, singing behavior likely affects FOXG1 gene expression. Although we did not have time to explore the potential for co-staining, it is predicted that the MeCP2 and FOXG1 genes regulate each other, and this interaction will be explored in future experiments.

3. Materials and Methods

3.1 Animals

The zebra finch songbirds were kept in cages, and usage was approved by the Louisiana State University Health Sciences Center (LSUHSC) Institutional Animal Care and Use Committee (IACUC). Male zebra finch songbirds were used for all experiments, and each bird was given an identification number at hatching.
The birds were housed at the LSUHSC with *ab libitum* access to food and all the adult birds were housed in single-sex group cages. The brains of only the male birds were collected as the female birds do not sing and get influenced by the male song. The nuclei develop to a greater extent in males than females and they are altered in size and organization during the critical juvenile learning period (Warren et al., 2010).

The bird diet consisted of seeds from Magnolia bird farms; however, specific food amounts were not limited. Breeding pairs and juvenile birds of 60 days or younger received ABBA seed supplements. The expression in the main songbird nuclei such as Area X, HVC, RA, and LMAN was examined at specific time points, including 45 days and 100 days, for the juvenile and adult bird, respectively.

The birds were sacrificed at crucial reference points in song development and brains were cut either sagittally or frontally at a thickness ranging from 40-60 microns. The location and expression of the MeCP2 and FOXG1 genes within male zebra finch brains were determined using immunohistochemistry with MeCP2 and FOXG1 antibodies.

Imaging was performed using fluorescence microscopy with the 10x and 40x lenses through DAPI, FITC, and CY3 channels. Once the general expression patterns of the genes were determined between the 60-day and 100-day time points, staining was used to determine whether the genes were regulated by song behavior.
3.2 Perfused tissue and brain slice preparation

Birds were anesthetized and the sacrifice protocol followed previous ones of Dr. Li's lab (Shi et al., 2017). The injection site was sprayed with 70 percent ethanol and birds were anesthetized with 120-150 uL intraperitoneal 3 mg/mL ketamine hydrochloride and 1.5 mg/mL xylazine hydrochloride in 0.9 percent sodium chloride five to ten minutes before surgery. It was ensured that the bird was entirely anesthetized by gently tugging on its wings. If a response was detected, 20 more uL of anesthesia was delivered.

The bird was placed on a Styrofoam board with all four extremities pinned down and a median incision was made, opening the bird's abdomen. The sternum was held up and the diaphragm was cut from the center outwards while cutting either side of the rib cage. The right atrium was cut and the heart was held delicately with fine forceps to push 25-50 mL of PBS slowly (~2.5-3 MLS/min). To ensure that perfusion was working, the tissue experienced color fading. The solution was changed to 4 percent paraformaldehyde and was pumped through the bird to allow for general animal stiffening.

The head was then detached from the body using sharp scissors and the brain was removed for fixation. The brain was fixed in 4 percent paraformaldehyde by immersion in the solution for eight hours at 4°C. The fixed brain was transferred into 15 percent sucrose overnight at 4°C and the solution then became replaced by ~15 mL of 30 percent sucrose at 4°C before being cut in the cryostat and being used for staining.

Following perfusion and brain harvesting, the perfused brain sections were cut sagitally or frontally into 40 µm using a cryostat at -14 ºC. Isolated tissue sections that were not used were transferred to wells filled with PBS and stored at 4°C.
3.3 Immunohistochemistry

Animals were perfused and sliced sagittally or frontally. Brain slices were first rinsed six times for five minutes each with 0.1 M PBS on the shaker. Slices were placed in a blocking solution for an hour with normal goat serum, 10 percent Triton X-100, and 0.1M PBS. The incubation period followed overnight with either the primary MeCP2 or FOXG1 antibody at a concentration of 1:1000 at 4°C. We tested antibody concentrations and determined that a 1:1000 FOXG1 concentration is likely the most ideal condition; however, we continue to optimize the conditions used. The staining occurred on a 60-day bird to examine the pattern of FOXG1 expression. The gene expression was then compared to that of an adult bird, typically at around 100 days.

The primary antibody for the MeCP2 staining was the Sigma-Aldrich M6818 monoclonal Anti-MeCP2 antibody produced in mouse. The secondary used was the anti-mouse antibody. The Abcam FOXG1 antibody was produced in rabbit and used the goat anti-rabbit Cy3 (red) secondary. The wells were then rinsed three times for ten minutes each in 0.1 M PBS.

The sections were incubated in the secondary antibody mixture for two hours covered on the shaker at room temperature. The tissues were washed twice for five minutes each with PBS, then underwent one five-minute DAPI wash at 1:1000 dilution before being washed three times with PBS. The sections were finally mounted one at a time on slides and coverslipped using a Slofade mounting medium without DAPI.

Imaging was performed through fluorescence microscopy. The 10x lens montage feature served to obtain a general image of the brain tissue. Such magnification allowed for the interpretation of how gene expression may vary across brain regions. The 40x lenses were then used to zoom into the song nuclei region and obtain cell-specific images. Staining effectiveness was determined through a comparison between DAPI and FITC channels for the MeCP2 gene. For
the FOXG1 gene, DAPI and CY3 channels were used. The specific areas examined through fluorescence microscopy were Area X and the HVC. We also considered whether or not there were signs of regional differences in gene expression between areas such as the cortical, sub-cortical, and striatal regions.

The imaging first occurred through the 10x lens using the montage feature to obtain a large frame of the entire tissue section. This extended view highlights general areas of heightened gene expression and regional differences in gene patterns. The imaging continued through the 40x lens, and focused on comparing images within the song nuclei and outside, to make note of any differences in cell size and density of expression.

Figure 3. Stepwise experimental procedure
A. Zebra bird perfusion
B. Brain cutting
C. Immunohistochemistry staining
D. Imaging through fluorescence microscopy
3.4 Singing versus Non-Singing Birds

The zebra finch songbirds know to remain silent in the dark, and therefore only sing in the light. To regulate non-singing behavior, birds are collected from their cages when it is dark, then the experimenter sits with them for an hour to ensure they are not singing. If they end up singing, they cannot be used for the non-singing group. For the singing bird group, the male bird is taken from the cage and then a female bird is placed next to the bird. Then, the experimenter marks how many times the bird sings within a certain time frame.

There are two types of zebra finch songbird song: directed and undirected. For the directed song, the male bird looks at the female bird and sings to her. The song in these instances is stereotypical and the bird does not change its song. For the undirected song, the bird is standing and singing and there is variability in the song.

3.5 Gene Expression-Neurons Specificity

Through examining the expression of the FOXG1 gene within the song nuclei, we could obtain the types of neurons in which the FOXG1 gene is expressed in. Within Area X, it has been determined that there are primarily spiny neurons. It is known that Darpp32 stains spiny neurons (Robra & Thirumalai, 2016). Further experiments plan to examine whether the FOXG1 gene is expressed in spiny neurons, and co-staining of the antibody will take place in Area X using Darpp32 as a marker. If interactions between the MeCP2 and FOXG1 genes are present, they would have to be expressed in the same neuron type.
3.6 Quantification

Although the quantification is at its initial stages and we are still collecting qualitative data, quantitative analysis will occur in future iterations of the experiment. Through the Image J software, the fluorescence staining was quantified by relying on the signal intensity of the cells expressing either the MeCP2 or FOXG1 gene within the song nuclei. There is also the possibility of using Western blotting to determine the number of proteins expressed within certain areas of the brain tissues. Again, we are currently still in the data collection phase and are experimenting to determine an optimal way to quantify the results obtained.

4. Results

The results shown below were based on qualitative measurements obtained through imaging using fluorescence microscopy and quantitative measurements through ImageJ. The images were taken using 10x and 40x lenses to observe gene expression patterns in the song nuclei. Basic signal intensity quantification methods were also used. Further quantification techniques are underway and aim to supplement qualitative analysis.

a. Juvenile vs Adult zebra finch songbirds

MeCP2 Gene Expression

The DAPI column indicates the DAPI staining, which targets cell nuclei. DAPI is therefore used as a positive marker, and the location of gene expression within cells can be compared to that of the DAPI channel. The FITC column shows MeCP2 staining within cells. Figure 4 shows heightened MeCP2 expression in the striatum region of the juvenile brain tissue. There was less
MeCP2 expression in the striatum region of the adult brain, but differential expression between the striatal and sub-cortical region was observed.

![Figure 4. MeCP2 gene expression in Area X of juvenile and adult bird brains](image)

**Figure 4. MeCP2 gene expression in Area X of juvenile and adult bird brains**

*Figure 4* compares the expression of the MeCP2 gene (1:1000) in the juvenile (Bird ID: 99R) vs. adult bird (Bird ID: 114R)

- Row A shows the 10x Montage (8x10 view) – scale bar of 100 µm
- Row B shows the 40x view of Area X – scale bar of 10 µm
- Row C shows the 40x view of outside Area X – scale bar of 10 µm

The MeCP2 gene expression in *Figure 5* shows different expression between the juvenile and adult bird brain. In Area X of the juvenile bird, the MeCP2 expression appeared to be in the cytoplasm. In the adult brain, gene expression was marked in the nuclei of the cells. This can be seen through the overlapping of the DAPI and FITC channels in *Figure 5*. For the juvenile bird, in the third column, in Area X, there is more green staining around the cells, whereas the green FITC staining for the adult Area X cells fills the entirety of the cells. Also, for the juvenile bird, a greater difference in gene expression was observed in Area X when compared to outside Area X but still in the striatal region. Specifically, cytoplasmic gene expression was evident in Area X of
the juvenile bird, but general MeCP2 expression in the cell nucleus was observed right outside Area X.

Figure 5 compares the expression of the FOXG1 gene (1:1000) in the juvenile (Bird ID: 99R) vs. adult bird (Bird ID: 114R)
- Row A shows the 40x view of Area X – scale bar of 10 µm
- Row B shows the 40x view of outside Area X – scale bar of 10 µm
FOXG1 Gene Expression

Upon general comparison between a juvenile and adult bird, the FOXG1 gene expression was heightened in the juvenile bird. Additionally, the expression varied in different areas of the juvenile brain. There was higher FOXG1 expression in the striatal (region 3 in Figure 6) and cortical (region 1 in Figure 6) regions, and a lower expression in the sub-cortical region (region 2 in Figure 6). In the adult bird shown on the right of Figure 6, a more uniform expression pattern was seen and there is a faint differential expression between the cortical, sub-cortical, and striatal regions. The difference was mostly seen between the striatal area and the rest of the brain tissue, with less of a heightened expression in the cortical region.

Figure 6. FOXG1 expression in juvenile and adult bird brains

Figure 6 compares the expression of the FOXG1 gene (1:1000) in the juvenile (Bird ID: spearmint 87) vs. adult bird (Bird ID: green 38)
- Row A shows the 10x Montage (12x12 view) – scale bar of 100 µm
- Row B shows the 40x view of Area X – scale bar of 10 µm
- Row C shows the 40x view of outside Area X – scale bar of 10 µm
In the juvenile bird, greater expression of the FOXG1 gene was noticed in Area X (row B) when compared to outside an area outside Area X but still remaining in the striatum (row C) as seen in Figure 6. In the adult bird, however, such a result was not as clear. There appeared to be similar FOXG1 gene expression in both Area X and outside Area X in the adult bird brain.

Figure 7. 40x view of FOXG1 expression in Area X of juvenile and adult bird brains

Figure 7 compares the expression of the FOXG1 gene (1:1000) in the juvenile (Bird ID: spearmint 87) vs. adult bird (Bird ID: green 38)
- Row A shows the 40x view of Area X – scale bar of 10 µm
- Row B shows the 40x view of outside Area X – scale bar of 10 µm

In terms of gene expression within Area X, the FOXG1 gene expression was greater in the juvenile bird than the adult bird brain, seen through Figure 7. In Figure 8, mean pixel intensity for quantification showed higher expression for the juvenile bird than for the adult bird, as the intensity was approximately 32 and 23 pixels respectively.
Figure 8. Signal intensity quantification for FOXG1 gene expression in Area X

In Figure 9, the expression pattern across the entirety of the juvenile spearmint 87 bird was shown. In row A of the Figure 9, the differential expression of the FOXG1 gene is highlighted. Specifically, there is increased gene expression in the LMAN region, pointed to with the white arrow. Rows B and C, showed a narrowed view of the area using both Montage and regular 10x microscopy features. In row B, the 10x Montage suggests a ball-like shape, LMAN, with overall heightened gene expression.
Figure 9. FOXG1 expression in the LMAN of a juvenile bird brain

Figure 9 shows the expression of the FOXG1 gene (1:1000) in the juvenile (Bird ID: spearmint 87)

- Row A shows the 10x Montage (12x12 view) – scale bar of 100 µm
- Row B shows the 10x Montage view of LMAN – scale bar of 100 µm
- Row C shows the 10x view of LMAN– scale bar of 100 µm

Through imaging, we searched for the HVC, specifically within the cortical region. Using another subset of birds, the HVC pattern of FOXG1 expression was observed. The outline of the HVC was more clearly defined in the adult bird, with the protrusion out of the general outline of the brain tissue highlighted in row A of Figure 10.
Figure 10. FOXG1 expression in the HVC of juvenile and adult birds

Figure 10 shows the FOXG1 gene expression (1:1000) in the juvenile (Bird ID: V20) and adult bird (Bird ID: 114)
- Row A shows the 10x Montage (12x12 view) – scale bar of 100 µm
- Row B shows the 40x view of HVC – scale bar of 10 µm
- Row C shows the 40x view of HVC – scale bar of 10 µm

In Figure 10, the juvenile bird demonstrated staining in more large cells and fewer small cells. In the adult, there was not a preference for large cells. Additionally, in the DAPI channel of the juvenile bird, the DAPI cell positive control staining showed smaller cells overall in size compared to that for the juvenile. The adult bird also appeared to have signs of cell clustering, with the smaller cells clumping together when imaging using the 40x lens shown through Figure 11. In the juvenile bird, there was a scattered pattern with some clustering; however, as the bird gets older, we noticed that the clustering also increases in pattern.
**Figure 1.** 40x view of FOXG1 expression in the HVC of juvenile and adult birds

**Figure 1** shows a 40x view of the expression of the FOXG1 gene (1:1000) in the juvenile (Bird ID: V20) and adult bird (Bird ID: 114) in the HVC region.

**b. Singing vs non-singing zebra finch songbirds**

**I. MeCP2 Gene Expression in Area X**

The singing bird, light blue 42, and the non-singing bird, violet 48, seen in **Figure 12**, were cut frontally and the staining patterns obtained are shown in **Figure 12**. Through the DAPI channel, the cell nuclei were stained as a positive control and through the FITC channel, the MeCP2 gene expression was observed through cell staining.

**Figure 12.** MeCP2 gene expression in a singing vs. non-singing bird

**Figure 12** compares the expression of the MeCP2 gene (1:1000) in the singing (Bird ID: light blue 42) vs. non-singing bird (Bird ID: violet 48)
- Row A shows the 10x Montage (12x12 view) – scale bar of 100 µm
- Row B shows the 40x view of Area X – scale bar of 10 µm
- Row C shows the 40x view of Area X – scale bar of 10 µm
Using the 10x Montage feature to show the entire frontal section, both the singing and non-singing bird section showed enhanced expression in the striatal area, where Area X is found. For the singing bird, within the area of the striatum containing Area X, the gene expression signal was slightly weaker and a circle of lower gene expression was found in Area X. Within the striatum of the non-singing bird, MeCP2 gene expression in Area X stood out through the FITC channel. The quantification through mean pixel intensity in Figure 14 showed an expression of 39.5 for the singing bird and 45 pixels for the non-singing bird.

**Figure 13.** Closer look at the MeCP2 expression in singing (Bird ID: light blue 42) vs. non-singing bird (Bird ID: violet48)
II. FOXG1 Gene Expression in Area X

For the FOXG1 gene, the gene expression also was examined in the singing vs. non-singing bird. As seen through Figure 15, there was greater FOXG1 gene expression in the singing bird. As seen through row B of Figure 15, there are more cells in the CY3 channel that have gene expression in the nucleus, overlapping with the DAPI channel.

Figure 15 compares the expression of the FOXG1 gene (1:1000) in the singing (Bird ID: light blue 42) vs. non-singing bird (Bird ID: violet 48)
  - Row A shows the 10x Montage (12x12 view) – scale bar of 100 µm
  - Row B shows the 40x view of Area X – scale bar of 10 µm
The expression of the FOXG1 gene was heightened in the Area X region of the song circuitry. Upon comparison to an area outside of Area X, there were more nuclei-stained cells within the CY3 channel of the field within Area X than outside. In Figure 16, there is more overlap between the gene expression in the DAPI and CY3 channels than there is for the singing bird outside of Area X.

![Figure 16. FOXG1 gene expression in Area X of a singing vs. non-singing bird](image)

**Figure 16** shows a closer look at the comparison between the 40x view of Area X for the singing bird compared to a region outside of Area X for the same bird.
5. Discussion

The results preliminarily indicated that for MeCP2 gene expression within Area X, juvenile birds had expression in the cytoplasm and adult showed expression in the cell nuclei. The MeCP2 gene organizes chromatin structures inside the nucleus (Damen & Heumann, 2013). It is speculated that age can cause MeCP2 gene translocation from the nucleus to the cytoplasm or there can be condensation of the MeCP2 gene in the nucleus. Given the higher level of staining in the cytoplasm of the cells in the juvenile sections, it is possible overall MeCP2 gene expression varies in location within individual cells as the bird transitions from the end of sensory learning at 60 days to adulthood at approximately 100 days. Further analysis is necessary to determine whether and how the translocation affects the interaction of the MeCP2 gene with other transcription factors, including the FOXG1 gene.

The results also preliminarily found that the FOXG1 gene is expressed in the zebra finch song circuitry, specifically in Area X, LMAN, and HVC, the main nuclei of the song learning circuitry. Greater FOXG1 gene expression in these areas was found in the juvenile bird compared to the adult bird. The results demonstrated that FOXG1 expression is likely to be developmentally regulated and increases when the bird is developing a song pattern during the sensory learning period the juvenile bird experiences.

The FOXG1 expression was expressed differentially between brain regions and enriched in the song nuclei. It was heightened in the striatal areas, including Area X as well as the cortical region, which comprises the HVC. The middle (sub-cortical) region, which was the area with minimal song nuclei, showed less gene expression. One area that did show heightened expression in the juvenile bird for FOXG1 expression was LMAN, which is also part of the song circuitry. Our data therefore indicate that the FOXG1 gene is involved in the zebra finch song circuitry. As such,
FOXG1 gene mutations likely lead to language impairment, particularly during a critical song learning period, which requires further analysis.

Within the HVC, the juvenile had FOXG1 expression in more large cells and fewer small cells. In the adult, however, there was not the preference for large cells. When birds learn to sing the neurons come into the HVC, and a juvenile bird is still in the crucial song learning period. Past studies have examined the projections of neurons originating from the RA and Area X to the HVC of the zebra finch bird brain. The experimentation focused on looking at systematic differences between gene expression in neurons either undergoing spontaneous replacement or not in the adult zebra finch brain. The study used neuronal tracers, laser capture microdissection, along with RNA profiling (Lombardino et al., 2005). The cell bodies from backfilled HVC-RA and HVC-Area X neurons were labeled through fluorescence then microdissected using a laser capture microscope. Overall, the study found that in birds, UCHL1 gene expression increased with singing behavior in replaceable HVC neurons, but this was not the case in the nonreplaceable neurons (Lombardino, 2005). Singing also pointed to a greater life expectancy for the HVC’s replaceable neurons. Because the large cells come to the HVC first, then the smaller cells are added, there is possible explanation for the clustering effect observed in the adult bird.

In the singing versus non-singing bird, the MeCP2 expression was heightened in the striatum of the singing bird but there was a decreased expression within Area X. In non-singing brain, gene expression was uniform throughout the striatum. There is a possibility that singing may downregulate MeCP2 gene expression in Area X given the results obtained. With the FOXG1 gene expression in the singing versus non-singing bird, the singing bird had a higher FOXG1 expression. The heightened expression of the FOXG1 gene in Area X suggests that the gene is
regulated by singing behavior and may be increased when the male birds develop their song during sensory development.

The results obtained are relevant to human studies because Area X can be paralleled with the human basal ganglia. By better understanding how gene expression occurs in the song nuclei of the zebra finch songbird, we can create hypotheses about gene expression in the human brain. The zebra finch bird model is a unique animal because we know its neurocircuitry and the process of song learning mimics that of human babies. Just as humans have a critical period of language development, songbirds must learn their song within a certain time frame of their lifespan. Birds learn to sing from other tutor birds just as children learn from other individuals around them. The MeCP2 and FOXG1 genes are both nuclear proteins that regulate gene expression, so we can better understand how upregulation or downregulation might apply to human neurodevelopmental disorders at critical time periods and in speaking vs. non-speaking criteria.

6. Limitations

The main limitation was the small sample size. Given that only a few birds were used for the experiment, it is necessary to repeat the trials and note deviances in results and analyses. Most studies use approximately five to six birds, however our own imaging typically only used one bird for every condition, such as one juvenile bird and one adult bird. To our knowledge, our study was the first time that the FOXG1 gene has been examined in the zebra finch songbird model. There were many instances of troubleshooting where staining or imaging conditions did not work; thus, these results represent only a preliminary analysis, and we are working to finetune our methods.

Another limitation was the minimal amount of quantitative data. Given the recency of research in such a model with the FOXG1 gene, our study focused on qualitative assessments to
better understand gene expression throughout the song circuitry, and specifically in the song nuclei. In the future, we plan to experiment with greater samples for intensity quantification. We also plan to use Western Blotting to understand protein expression in the regions of interest. We were not able to include any statistical analyses as we did not have sufficient quantitative data. Future studies should include such analyses to determine if the results present statistical significance.

The age points used were 60 days for a juvenile and 100 days for an adult bird. In future staining procedures, additional age points should be included. For example, the 45-day age point is a crucial additional age point to examine in future studies to determine whether there are any changes in gene expression between the time of vocalization and when the sensory learning window closes. Within the sections, varying thickness of samples was another limitation as some samples had previously been sliced and kept in the freezer whereas others were removed from the freezer. Future studies must maintain identical conditions for brain slicing and preservation to better compare staining and imaging results obtained.

When conducting studies with adult birds, it is unknown whether they are singing or non-singing birds. It is necessary to conduct multiple trials with different adult birds to avoid any confounding variables in gene expression that could be due to singing behavior. An additional control would therefore be necessary when selecting birds for the juvenile versus adult gene expression comparison.

Overall, follow-up studies are necessary to validate the data obtained. Specifically, the same staining procedures must be repeated to determine how reproducible the results are. These methods must be repeated with more than one set of birds for the juvenile and adult groups as well as with the singing versus non-singing groups.
In future iterations of the MeCP2 and FOXG1 gene analysis, we plan to look for signs of co-regulation between the MeCP2 and FOXG1 genes. Due to the small sample size and the novelty of the FOXG1 gene research, before analyzing co-regulation, we must first develop more robust conclusions for the MeCP2 and FOXG1 gene expression patterns in juvenile, adult, singing, and non-singing zebra finch songbirds.
References


https://doi.org/10.1186/s12864-018-4578-0


https://doi.org/10.3390/biom11010075


https://doi.org/10.7554/eLife.29087


