THE UNIVERSITY OF CHICAGO

HOW JUVENILE ZEBRA FINCHES LEARN SONG: INVESTIGATING THE EFFECTS OF AGE, GENETIC SEX, AND ACUTE SONG EXPOSURE ON MOLECULAR MECHANISMS OF SENSORY SONG LEARNING

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“In the fell clutch of circumstance

I have not winced nor cried aloud.

Under the bludgeonings of chance

My head is bloody, but unbowed.”

- *Invictus* by William Henley

“Time held me green and dying

Though I sang in my chains like the sea.

- *Fern Hill* by Dylan Thomas

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Abstract

Social learning is an important part of development because it is a way that individuals can acquire patterns of behavior appropriate for the environment. Often, socially-learned behavioral patterns persist throughout the lifespan, making developmental learning an essential component to understanding adult behaviors. For example, in zebra finches (*Taeniopygia guttata*) both juvenile males and females form memories of song they hear from adult males and these memories have behavioral consequences for both sexes as adults. However, it is unknown if juvenile males and females learn song in the same way: adult males learn to sing, while adult females do not. In this dissertation I have investigated different molecular signatures of learning during development to try to elucidate whether juvenile male and female zebra finches are capable of sensory song learning in the same way.

The existing literature investigating known molecular mechanisms of learning in juvenile male and female zebra finches is inconclusive. This is largely due to the imbalance of information we have about sensory song learning between juvenile males and females – we know much more about males than we do females. Given that we know long-term memory formation requires that experience initiates new protein synthesis, my approach was to measure a molecular indicator of protein synthesis and a molecular regulator of mRNA available to be translated into protein across a known window of sensory song learning in both juvenile male and females. I did so in a way which would allow me to account for age, sex and acute song experience - all factors that can influence learning throughout development.

In Chapter 1, I reviewed the current literature and provided relevant background to ground the reader in the experiments which make up my doctoral work. In Chapter 2, I investigated a molecular indicator of new protein synthesis during the sensory song learning period of development in juvenile males and females. I used a counter-balanced design to compare birds with and without acute song experience across sex at different ages spanning the sensory song learning period of development. The results from this study suggest that one molecular indicator of sensory song learning in males may be different from that of females during this period of development. In Chapter 3, I designed an experiment to explore the abundance of a molecular mechanism which is a potential regulator of sensory song learning in juvenile males and females during development. In this chapter I described the experiment, the challenges that I faced in running the experiment, and the conclusions I drew from this experience. In Chapter 4, I described an experiment I designed in order to answer the questions posed in Chapter 3 this time using a different technique. I layout the methods and the results I expected to see using this technique. In Chapter 5, I summarized the main conclusions from my doctoral work and the potential future directions. Altogether, my work helps advance questions focused on the relationships between molecular mechanisms of social experience and behavior and how regulatory mechanisms and indicators of new protein synthesis can be utilized to characterized learning ability.

Chapter I: Introduction

Developmental Learning

Learning is defined as acquiring new or modifying existing knowledge through experience or study; it is important because learning can lead to changes in an individual's behavior. Throughout an individual's life, learning serves different functions. During development, learning is a way for individuals to acquire patterns of behavior appropriate for their environment: behaviors which will often persist throughout the lifespan. Learning as an adult can be used for individual identification, establishing and maintaining relationships, and for defense. Developmental and adult learning are interconnected because behaviors learned early in life often heavily influence adult behaviors. For example, when an eyelid is sutured shut during development the result is functional blindness in that eye, even though there is nothing structurally wrong with the eye - the retina remains entirely functional following the reopening of the eye at a later date (Hubel and Wiesel 1964).

Sensitive Periods

There are periods of time during an individual's development when experiences can have particularly strong and enduring effects on brain structure and behavior due to increased neural plasticity, known as sensitive periods (Bornstein 1989; Knudsen 2004; Uliana et al. 2021). There are three hallmarks of a sensitive period for learning: first, a defined period during development with a known onset and offset; second, a salient stimulus; and third, sufficient neural maturation to process and learn from the stimulus (Knudsen 2004; Fagiolini and Hensch 2000). In theory, the onset of a sensitive period can either be age-, experience-, or age and experience-dependent (Knudsen 2004). The effects of experience with the salient stimulus during this limited period of

development include changing brain architecture (Knudsen 2004). A subset of sensitive periods, known as Critical Periods, end when the individual has received enough experience from the salient stimulus. The accumulation of experience strengthens the appropriate neural circuits, because the mechanisms that were responsible for the heightened state of plasticity operate with much lower efficiency (Knudsen 2004). Lack of experience with the salient stimulus during Critical Periods does not permanently and irreversibly affect development, but rather can delay the opening of the sensitive period. This may be because there is a lack of accumulated experience to produce the experience-dependent changes in the epigenetic mechanisms that may regulate sensitive period plasticity (Knudsen 2004; Kelly et al. 2018; Hernandez and Abel 2008; Davis and Squire 1984).

Human Speech Example of Sensitive Period

The acquisition of human language can be used to better understand the behavioral changes associated with the progression of a sensitive period. Human language consists of a series of sensitive periods; perceptual attunement to the properties of native language and syntax (Werker and Hensch 2015; Ruben 1999; Werker et al. 1981). In the sensitive period for phoneme discrimination, the ability to discriminate many of the consonant distinctions is present prior to listening experience, however, the effect of listening experience is to maintain (and sharpen) sensitivity to those distinctions used in the native language and to show a decline in discrimination of nonnative ones (Werker and Hensch 2015; Hurford 1991; Werker et al. 1981; Neville et al. 1992; Kuhl 2010; Knudsen 2004; Werker and Tees 2005). Babies learn to babble and ultimately to speak by mimicking the sounds they hear from their caretakers. Not only is speech a behavior the babies will depend on for the rest of their lives, but learning language is also a way for babies to pick up the vernacular appropriate for their immediate environment.

Language and other complex behaviors depend on a wide range of specialized sensory and motor skills. An understanding of how these specialized skills is reflected throughout the progression of a sensitive period can be understood through the concepts of receptivity and responsivity. Prior to the opening of a sensitive period, an individual may be receptive to an experience or stimulus. This is when an individual is using its sensory skills to receive information from the environment. At the opening of a sensitive period, an individual has the ability to "respond" to an experience or stimulus. This "response" comes in the form of internal sensorimotor learning. Sensorimotor learning is defined in this dissertation as a specific form of learning defined as the fine tuning of connections between the sensory system that interprets external environmental signals and the motor system that will eventually contribute to a physical response.

Although sensitive periods are reflected in behavior, they are in fact a property of neural circuits and the molecular mechanism that govern neural plasticity. Therefore, to define sensitive periods and to explore why they occur and how they might be manipulated, we must think about what is happening in these circuits on a molecular level.

Molecular Mechanisms of Learning and Memory

Across several species, long-term stable memory formation requires that experience initiates new protein synthesis. (Smalheiser et al. 2010; Griggs et al. 2013; Rajasethupathy et al. 2009; Litvin and Anokhin 2000; Ponomarenko and Kamyshev 1997; Ahmadiantehrani and London 2017; London et al. 2009; Matthies 1989; Davis and Squire 1984). New protein

synthesis is the process of mRNA being translated into proteins (**Figure 1-1**). There are many direct and indirect mechanisms that can indicate new protein synthesis is occurring, as well as mechanisms which either positively or negatively affect the rate of new protein synthesis. One example of a mechanism that demonstrates experience-dependent changes throughout development with known functions in facilitating learning and memory is the activity of a specific epigenetic modulator called microRNA (miR). miR regulate which and how many of each mRNA are available for translation.

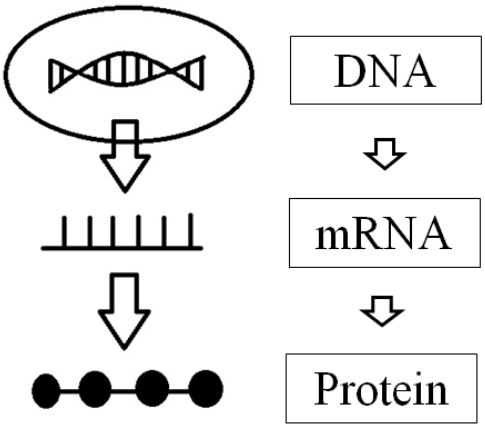

Figure 1-1 The process of new protein synthesis.
DNA is transcribed into mRNA, which is then translated into protein. DNA consists of two strands of deoxyribonucleic acids linked together and wound around each other into a double helix shape found within the nucleus of the cell (the bowtie figure within the circle at the top). The four nucleotides of DNA are Adenine (A), Guanine (G), Cytosine (C), and Thymine (T). DNA is then reverse transcribed to create a single strand of nucleotides which will become mRNA (the single strand outside of the circle). The four ribonucleic acids of RNA are Adenine (A), Guanine (G), Cytosine (C), and Uracil (U). mRNA is produced within the nucleus and then transported into the cytoplasm for translation. During translation, the mRNA sequence is used to determine which amino acids will make up the protein (the line with circles). Amino acids are organic compounds that contain both amino and carboxylic acid functional groups. These functional groups interact with different functional groups from other amino acids causing the protein to fold in certain ways. The folding of the protein creates secondary and tertiary structure, which is why proteins are depicted here as connected circles rather than in a strand like DNA and mRNA.

Epigenetic Modulators

The epigenome is a collection of molecular modifications that regulate gene expression, while not altering the genomic sequence. There are several distinct epigenetic modifications, including DNA methylation and post-translational histone modification, each of which can direct the transcriptional regulation of various suites of genes (Surani et al. 1993). Epigenetic modifications operate throughout a lifetime to regulate gene expression in the brain. (Shang and Bieszczad 2022). Epigenetic modulators are the factors that influence the activity and/or localization of these epigenetic modifications. Epigenetic modulators can be affected by environmental factors, such as chemical and physical stressors like pollutants and radiation, providing a mechanistic link between the genome and what is happening in an organism's environment (Loscalzo and Handy 2014).

Non-coding RNAs are one such class of epigenetic modulators. Non-coding RNAs are functional RNA molecules that are not translated into proteins and are classified by transcript size (Wei et al. 2016). There are two main classifications, long non-coding RNA (lncRNA) and small non-coding RNAs (sncRNA). lncRNAs are involved in numerous biological functions and regulate gene expression through a diverse array of mechanisms (Gil and Ulitsky 2020; Quinodoz and Guttman 2014). They also display temporal, spatial and cell-type specific expression in the brain (Goff et al. 2015; Butler et al. 2019; Kadakkuzha et al. 2015). For example, lncRNA in hippocampal neurons is required for cAMP-dependent structural plasticity of dendritic spines in hippocampal neurons, influencing synaptic plasticity and likely learning and memory(Wang et al. 2016, Ye et al. 2011, Grinman et al. 2019). sncRNAs, such as small interfering RNAs (siRNAs) and microRNAs (miRs), have been shown to have similar functions

as lncRNA (Wei et al. 2016). miRs are the most studied sncRNA in the context of epigenetic modulation (Wei et al. 2016). miRs regulate which and how many of each mRNA are translated into protein via targeting specific mRNA for translational repression and/or degradation. miR regulate approximately 60% of protein-coding genes via these mechanism (Valinezhad Orang et al.2014; Vasudevan et al. 2007; Lee et al. 1993; Reinhart et al. 2000; Bartel 2004).

miRs can act as epigenetic modulators by positively or negatively regulating the rate of histone deacetylase (HDAC) catalyzed reactions. HDAC is an enzyme that removes the acetyl group from histone proteins on DNA, making the DNA less accessible to transcription factors and decreasing the probability of transcription (Seto and Yoshida 2014). miRs regulate HDAC-catalyzed reactions via the same mechanisms used in regulating mRNA translation (Kwa and Jackson 2018; Sato et al. 2016). For example, one miR, miR-206, is predicted to target, a specific HDAC, HDAC4 (Bourassa and Ratan 2014). miR-206 knockout mice had increased HDAC4 protein levels but not HDAC4 mRNA, which suggested that miR-206 regulates HDAC4 via mRNA degradation rather than transcriptional repression (Bourassa and Ratan 2014). This means that miR-206 binds to HDAC4 mRNA and prevents the translational machinery from translating the HDAC4 mRNA into protein.

What Factors Influence Learning During Sensitive Periods

One process that influences developmental learning is maturation. Maturation is the process of reaching an advanced stage of physical, mental and emotional development. As an organism matures, its brain grows in overall size and becomes increasingly organized and interconnected. The brain becomes more organized as specific areas, such as the forebrain,

midbrain, and hindbrain develop. The brain becomes more interconnected as synaptic connections are modified. Synaptic connections that are less used become weaker and ultimately perish. Synaptic connections that are used frequently become stronger and increase in number. The presence of organized and interconnected networks of neurons are important for the formation of memories and the ability to link new learning to previous learning (Braus 2004; Mujaway et al. 2021). Experience-dependent strengthening of synaptic connections helps establish neural networks which are foundational for forming the memories that support developmental learning.

We can leverage what is known about the molecular mechanisms of learning and memory to investigate how age and experience influence the encoding of experiences necessary for establishing adult behavior. To do this, we can take advantage of an animal model, specifically the zebra finch (*Taeniopygia guttata)*, in which both sexes learn during a period of development and as adults have sexually dimorphic behavioral patterns.

Zebra Finch Sensory Song Learning as a Model System

The formation of song memories in juvenile males and females has behavioral consequences for both sexes in adulthood; however: adult male zebra finches learn to sing, while adult females do not. In juvenile males, early memories of song are important for the male to develop and sing a stereotypical zebra finch song as an adult (Eales 1985; 1987; Bohner 1983; 1990; Slater et al. 1991; Roper and Zann 2006; Chen et al. 2017). The song an adult male sings emerges after a developmental process of coordinated sensory and motor functions. The foundation of song learning is sensory learning. Sensory learning is the process in which the

juvenile forms an auditory memory of an adult "tutor" bird's song. Through a process of sensorimotor error correction, the juvenile uses his memory of the tutor's song to guide his initial innate, immature vocalizations into an adult, stereotyped song structure that largely resembles that of the tutor's.

Sensory song learning in male zebra finches is a process that happens within a sensitive period during development. The sensitive period of development opens at post-hatch day 30, defined as P30, and has a song experience-dependent closure at around 65 days post-hatch, or P65 (Bohner 1983; 1990; Slater et al. 1991; Roper and Zann 2006; Adret et al. 2012; Chen et al. 2017; Braaten 2010; Eales 1985; 1987; Morrison and Nottebohm 1993) (**Figure 1-2**). Even though juvenile males interact with adult tutor males prior to P30, it is between P30 and P65 where juvenile males form an auditory memory of the tutor's song (Adret et al. 2012; Bohner 1990; Eales 1985; 1987; Roper and Zann 2006; Morrison and Nottebohm 1993; Slater et al. 1991) (**Figure 1-2**). If however, a juvenile male is isolated from song from P30 to P65, he can

learn song after the typical closure of the sensitive period and this song will be patterned based on the tutor he experiences after P65 (Eales 1985; 1987; Morrison and Nottebohm 1993).

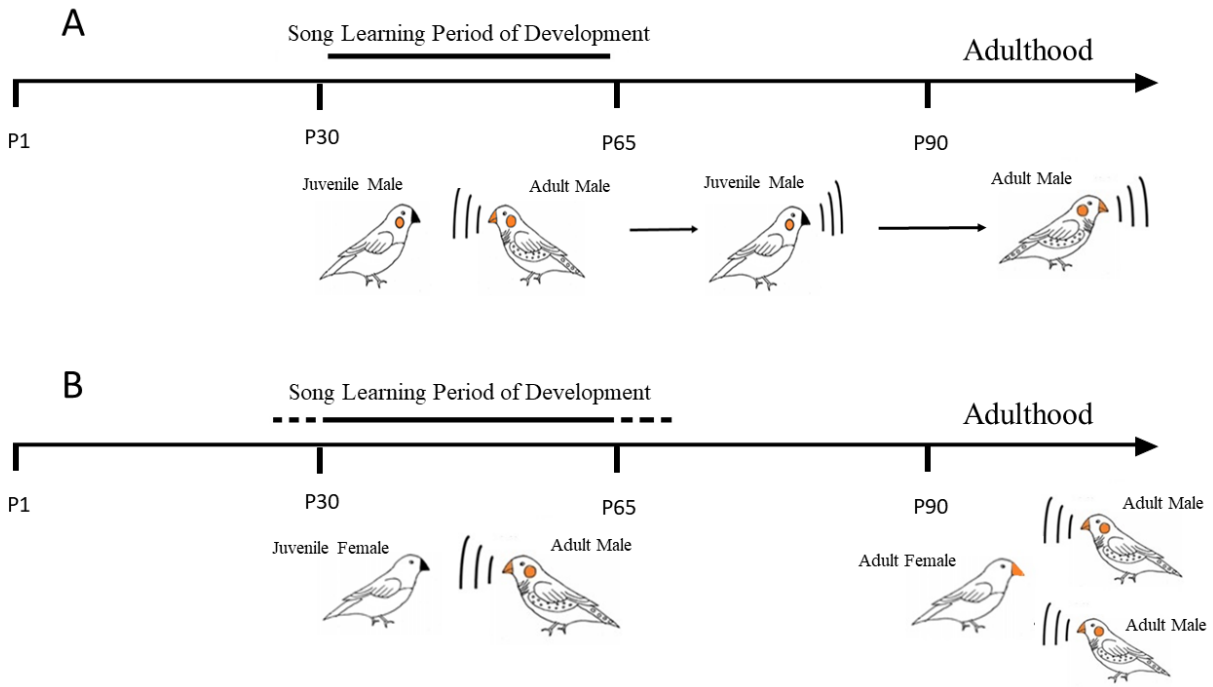

Figure 1-2 Timeline of sensory song learning during development and adult behavioral consequences in males and females.
Sensory song learning in juvenile males (A/top) and juvenile females (B/bottom) occurs during overlapping periods of development. Sensory song learning in juvenile males (black beak with orange cheek patch) normally occurs between post-hatch day 30, P30, and P65 (denoted by the black bar labeled as song learning period of development).. During this time, juvenile males create a memory of an adult male tutor's (orange beak with orange cheek patch) song (denoted by the three cascading semicircles) and they use this memory as a template to develop their own song. The juvenile male's song becomes crystalized by adulthood (P90). Sensory song learning in juvenile females (black beak with no cheek patch) occurs between P25 and P70, but the exact onset and offset of this period of development is not known (denoted by the dashed black line labeled as song learning period of development). Females create a memory of a tutor's song and use this memory to inform mate choice and mate recognition as adults (orange beak but no cheek patch). The unlabeled black bar arrow denotes the timeline of development between hatch (P1) and adulthood (P90). The birds position beneath the unlabeled black bar arrow corresponds with the ages at which the depicted behaviors are occurring.

In females, the early memories of song formed as juveniles inform adult mate choice and mate recognition (Lauay et al. 2005; Williams et al. 1993) (**Figure 1-2).** Females reared without

exposure to adult male song show clear preferences for species specific song (conspecific) over different species song (heterospecific), but song experienced females show clear preferences for tutored over untutored male song (Braaten and Reynolds 1999; Lauay et al. 2005; Riebel 2000; Williams et al. 1993). The sensory song learning period of development in juvenile females is not as well defined as it is in juvenile males. A number of studies have demonstrated that song experience between P25 and P70 results in the establishment of song preference in adult females, however, the exact onset and offset of sensory song learning ability in juvenile females is unknown (Miller 1979; Clayton 1988; Clayton 1990; Riebel 2000; Riebel et al. 2002) (**Figure 1-2**). This suggests that, similar to juvenile males, females are capable of learning specific characteristics of song during similar period of development.

Neurobiology of Zebra Finch Song System

In male zebra finches, a number of brain regions are involved in birdsong learning, perception, and production. These specialized and interconnected brain regions are readily visually distinguished from surrounding brain regions and are grouped into pathways (Lovell et al. 2008; Nottebohm et al. 1976; Nottebohm and Arnold 1976). One is the posterior vocal motor pathway which is required for song production composed of the brain nuclei HVC (proper name) and the robust nucleus of the arcopallium (RA) (Nottebohm et al. 1976, Nottebohm and Arnold 1976) **(Figure 1-3).** The other is the anterior forebrain pathway, composed of the lateral magnocellular nucleus of the anterior nidopallium (LMAN), Area X, and the medial part of the dorsolateral nucleus of the anterior thalamus (DLM). This pathway plays important roles in sensorimotor learning and song plasticity (Bottjer et al. 1984; Brainard and Doupe 2000; Aronov et al. 2008; Doupe and Solis 1997; Scharff and Nottebohm 1991; Olveczky et al. 2011) **(Figure**

1-3). These two pathways are connected via projections between nuclei in each pathway – HVC projects to Area X and LMAN projects to Area X, RA, and the RA dorsal the archistriatum (Ad) linking the anterior forebrain pathway with the posterior vocal motor pathway **(Figure 1-3) (**Bottjer et al. 1989; Johnson et al. 1995). These connections only develop naturally in birds that learn to sing (Bolhuis and Gahr 2006; Mooney 2009; Brenowitz 1997; Doupe et al. 2005; Roberts et al. 2008).

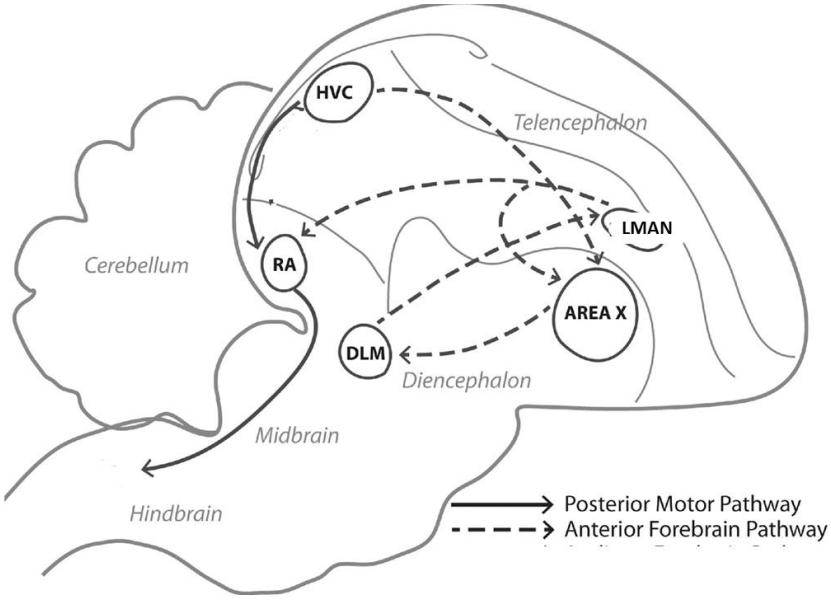

Figure 1-3 The brain nuclei which make up the song circuit in zebra finches.
The posterior vocal motor pathway is in the solid line. The posterior vocal motor pathway consists of HVC and RA. The anterior forebrain pathway is in the dashed line. This pathway consists of HVC, Area X, DLM and LMAN. Inputs from HVC project to Area X, then projections from Area X travel to DLM and projections from DLM travel to LMAN. The anterior forebrain pathway is connected to the posterior vocal motor pathway via projections from LMAN to RA, an area surrounding RA dorsal the archistriatum (Ad), and to Area X and projections from HVC to Area X. This figure is adapted from London 2019.

Neurobiology of Why Females Do Not Sing

It is important to first understand the similarities and differences of how the zebra finch brain is organized in males compared to females in order to comprehend why adult females do

not sing even though juvenile females form memories of song. The ability of juvenile males to form a memory of a tutor's song and use this memory as a template for the juvenile male's own song is the result of coordinated function across the song circuitry nuclei of the anterior forebrain and the posterior vocal motor pathways (Bottjer et al. 1985; Nottebohm, Kelley, and Paton 1982, Nottebohm and Arnold 1976). Females have the same brain regions; however, the LMAN, Area X, HVC, and RA are much smaller in females than in males (Nottebohm and Arnold 1976; Nixdorf-Bergweiler 1996; London et al. 2003). These differences in size do not emerge all at once and are thought to result in the lack of song production observed in females (Bottjer et al. 1984, 1985; Blair Simpson and Vicario 1990; Scharff and Nottebohm 1991; Nottebohm and Arnold 1976; DeVoogd and Nottebohm 1981; Gurney 1981; Nixdorf-Bergweiler 1996).

Although females do not sing, masculinization of their song nuclei via estradiol administration during development can result in song production in adulthood (Gurney 1982, Gurney and Konishi 1980, Simpson and Vicario 1991, Adkins-Regan et al. 1994). The song that estradiol treated females sing in adulthood is learned during development (Simpson and Vicario 1991). The ability to artificially create female birds who sing is not surprising given that in the common ancestor of modern songbirds, females sang (Odom et al. 2014). These data suggest that juvenile males and females may perform sensory song learning in the same way, but modern unmanipulated females do not have neural connections between song circuitry nuclei available to be strengthened by song experience (Nottebohm and Arnold 1976; Nixdorf-Bergweiler 1996).

Auditory Forebrain and Juvenile Sensory Song Learning

There is a third pathway within the song circuit which consists of auditory cortex-like regions that are involved in auditory processing and perception (Bolhuis et al. 2010; Jarvis et al. 2005; Mello et al. 2004; Mello 2004). The brain region in this pathway is the auditory forebrain. This brain region contains three distinct, highly-interconnected sub-regions; an area analogous to the mammalian primary auditory cortex (Field L), and two areas analogous to the mammalian secondary auditory and association cortexes, the caudomedial nidopallium (NCM) and caudomedial mesopallium (CM) (London and Clayton 2008; Yanagihara and Yazaki-Sugiyama 2016; Nottebohm and Arnold 1976; Ahmadiantehrani and London 2017; Phan et al. 2006; Gobes et al. 2010; Mello et al. 2004; Vates et al. 1996) (**Figure 1-4).** In adult males, projections initiating in Field L and CM travel to the HVC and RA to their adjacent processing areas of HVC and RA, HVC-shelf and RA-cup (not represented), respectively (Vates et al. 1996; Bauer et al. 2008; Theunissen et al. 2004). These projections provide an anatomical pathway for auditory information to become integrated with the motor production (Vates et al. 1996; Bauer et al. 2008; Theunissen et al. 2004) (**Figure 1-4).**

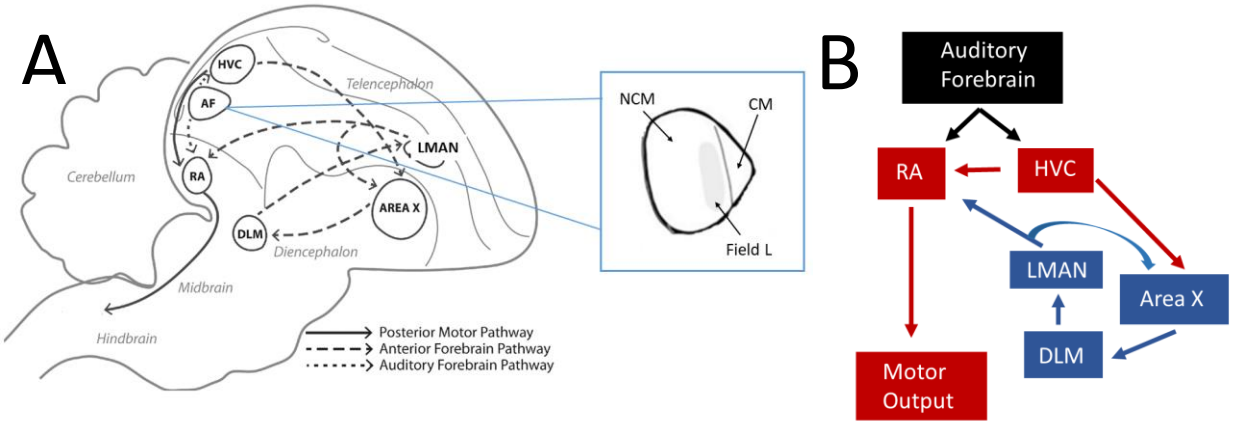

Figure 1-4 The interactions between the auditory forebrain and the song circuitry nuclei in male zebra finches.
The posterior vocal motor pathway is in the solid line. The anterior forebrain pathway is in the dashed line. The auditory forebrain is labeled AF. Panel A is neuroanatomical localization information of the various song circuitry pathways with the connections between brain nuclei. Panel B is how information flows from auditory stimuli through the song circuitry system. Panel A is adapted from London 2019.

Unlike other singing circuitry brain regions, the NCM, CMM, and Field L appear similar in size and structure in males and females (Krentzel and Remage-Healey 2015; Brenowitz 1997). However, even though there is a possibility of anatomical similarities between male and female auditory forebrain, there are known differences in neurophysiology and molecular responsivity of this brain region in males compared to females (Dagostin and Mello 2012; Yoder et al. 2014; Bailey and Wade 2003; 2005; Pinaud et al. 2008; Ahmadiantehrani and London 2017; Ahmadiantehrani et al. 2018).

This dissertation focuses on the NCM and CM brain regions. There is a great deal of evidence to suggest that these higher-order auditory processing regions play a role in the processing and memorization of songs (Bolhuis and Gahr 2006; Mello et al. 2004; Mello 2004; Hahnloser and Kotowicz 2010, London and Clayton 2008, Ahmadiantehrani and London 2018,

Mello et al. 1992; Mello and Clayton 1994, Bailey and Wade 2003; Jin and Clayton 1997; Stripling et al. 2001, Bolhuis et al. 2000; 2001; Terpstra et al. 2004; Phan et al. 2006, Gobes and Bolhuis 2007, Yanagihara and Yazaki-Sugiyama 2016). More is known about the function of the NCM and CM in juvenile male sensory song learning compared to juvenile sensory female song learning. In order to further characterize the function of the NCM and CM in female sensory song learning, we can compare the molecular signatures of learning within the NCM and CM across sex.

Learning and Memory within the NCM of Juvenile Males

In male zebra finches, the NCM was established as an important brain nuclei for song learning following the exploration of the expression patterns of *Zif-268*, more commonly known as *ZENK (zif268, egr-1, ngfi-a, krox24)*, within the brain in response to acute experiences with socially relevant (conspecific) and irrelevant (heterospecific song and tones) auditory stimuli (Mello et al.1992). Only conspecific song elicited an induction of *ZENK* within the NCM of adult males, suggesting that this area may represent the auditory discrimination and processing area in the male zebra finch brain (Mello et al.1992; Mello and Clayton 1994). These initial studies also found a lack of *ZENK* expression in the brain nuclei that control song production after acute song exposure (Mello et al. 1992; Mello and Clayton 1994). Follow-up work showed that the act of singing elicited an increase in *ZENK* in the brain regions that control song production, implying that the NCM was functionally separate from the motor aspects of the song circuitry nuclei (Jarvis and Nottebohm 1997; Jin and Clayton 1997). Within the NCM the *ZENK* response habituated specifically after repeated conspecific song exposure, suggesting that the rapid changes in *ZENK* response reflected a learned discrimination (Mello et al. 1995). The

NCM was further implicated as a brain region important for sensory song learning when it was shown that hearing conspecific song resulted in changes in *ZENK* expression that correlated with changes in electrophysiological responses within the NCM and with changes in behavior (Mello et al.1995; Chew et al. 1995; Chew et al. 1996; Stripling et al. 1997; Stripling et al. 2003; Thompson and Gentner 2010).

It was then investigated if these *ZENK* responses in the NCM were related to juvenile male sensory song learning. Studies focusing on the beginning of the sensitive period (i.e. P30) showed that at P30 a *ZENK* response was observed in the NCM but no response occurred in birds at P20 (Bailey and Wade 2003; Jin and Clayton 1997; Stripling et al. 2001, Roper and Zann 2006). Furthermore, P30-P40 birds that had been isolated from song (i.e., only raised with their mothers) showed no *ZENK* response to acute song exposure within the NCM (Jin and Clayton 1997). Studies focusing on the closure of the sensitive period (i.e. P65) showed that hearing the same song of the adult male which the juvenile male copied its own song from elicited a greater *ZENK* response within the NCM compared to the *ZENK* response after hearing other songs the juvenile male had been exposed to during development (Eales, 1985, Eales, 1987, Roper and Zann, 2006, Morrison and Nottebohm, 1993, Slater et al., 1991, Böhner, 1990, Böhner, 1983, Adret et al., 2012, Gobes et al. 2010). Consistent with these molecular findings, electrophysiological recordings showed that some neurons in the NCM selectively respond to conspecific song over heterospecific song either at a single-cell level or at a population level (Stripling et al. 2001; Schroeder and Remage-Healey 2021). However, it was also found that the selectivity is already present in P20 males (Stripling et al. 2001). This supports that the auditory

stimulus capable of inducing an electrophysiological response in NCM may not be sufficient to induce a molecular response.

The hypothesis that neurons within the NCM code for tutor song memory was originally suggested from studies in adult males (Bolhuis et al. 2000; 2001; Terpstra et al. 2004; Phan et al. 2006), but recent electrophysiological studies have shown that tutor song selective neurons are present at an early stage of song learning and that such selectivity is actually shaped through auditory experience of tutor song (Miller-Sims and Bottjer 2014; Yanagihara and Yazaki-Sugiyama 2016; Moore and Woolley 2019). Consistent with this, when adult males were re-exposed to the tutor song they copied as juveniles, the number of cells which expressed *ZENK* and *c-Fos* were proportional to the number of elements that the bird copied from its tutor's song (Bolhuis et al. 2000; 2001; Terpstra et al. 2004). A series of studies then showed that the NCM in juvenile males is required for tutor song memorization. A subset of NCM neurons showed highly selective auditory responsiveness to tutor song (Yanagihara and Yazaki-Sugiyama 2016). Also, lesions to the NCM impaired tutor song recognition, but did not affect song production or call discrimination (Gobes and Bolhuis 2007). Finally, it was shown that the NCM is necessary for sensory song learning through a series of inhibition and the constitutive activation experiments within the auditory forebrain during the sensitive period for sensory song learning. One of these studies showed that an infusion of an ERK inhibitor into the auditory forebrain prevented juvenile males from being able to accurately create a template of their tutor's song (London and Clayton 2008). ERK regulates the induction of *ZENK* within the auditory forebrain (London and Clayton 2008; Cheng and Clayton 2004). The infusion of the ERK inhibitor did not have an effect on acute song perception or song discrimination ability (London and Clayton 2008). The

next study discovered that within the NCM of P30, but not P23, males hearing conspecific song activated the mechanistic Target of Rapamycin (mTOR) signaling cascade (Ahmadiantehrani and London 2017). The activation of mTOR is known to be required for learned behavior in a variety of paradigms in adult rodents (Ahmadiantehrani and London 2017; Garza-Lombo and Gonsebatt 2016; Kazdoba et al. 2016; Giovannini and Lana 2016). This activation of the mTOR cascade was characterized by the increased in phosphorylation of S6 (pS6) (Ahmadiantehrani and London 2017). Further, both the infusion of an inhibitor and a constitutive activator of mTOR within the auditory forebrain during tutoring experiences significantly diminished the juvenile male's ability to copy the tutor's song (Ahmadiantehrani and London 2017). This suggested that balanced mTOR signaling is required for the onset of tutor song memorization in males (Ahmadiantehrani et al. 2018).

Learning and Memory within the CM of Juvenile Males

In one of the initial studies investigating the auditory processing regions of the zebra finch brain, it was shown that the CM contained neurons which responded to auditory cues and the percentage and selectivity of these neurons were comparable to what was found in the NCM (Muller and Leppelsack 1985). Studies investigating *ZENK* expression within the CM, referred to as the caudomedial hyperstriatum ventral (CMHV) in these studies, found that hearing conspecific song elicited an induction of *ZENK* within the CM of adult males (Mello et al. 1992; Mello and Clayton 1994). This suggested that the CM area may represent the auditory discrimination and processing area in the male zebra finch brain. It was then shown that hearing conspecific song resulted in *ZENK* induction and an increase in pS6 within the CM of P30 males, and these responses were similar to those seen in the NCM (Bailey and Wade 2003;

Ahmadiantehrani and London 2017). The requirement of the CM in tutor song memorization was shown through the ERK inhibition and bidirectional manipulation of mTOR study discussed previously in the context of the NCM, with the same results seen in the CM as the NCM (London and Clayton 2008; Ahmadiantehrani and London 2017).

Of important note, these studies also looked at the correlation between cannula placement within the auditory forebrain and song similarity score and found that scores were consistently lower for birds injected with the ERK or mTOR inhibitor compared to the controls at all cannula positions along both rostral and ventral axes (London and Clayton 2008; Ahmadiantehrani and London 2017). This could suggest that the activation of ERK and mTOR within the NCM is equally as important for tutor song memorization as is within the CM. It is important to note that while these publications did not reveal a substantial effect of cannula placement on tutor song copying, however, they did report that as the cannula location became more posterior, the song similarity score decreased and that a repeat of this experiment using smaller amount of drug targeted to specific auditory forebrain sub-regions could uncover an effect of spatial location on tutor song memorization. It is unlikely that in juvenile males both the NCM and the CM have the same function in facilitating sensory song learning even though both are required for tutor song memorization. While it has been shown that IEG expression is proportional to how well the juvenile copied its tutor's song in the NCM, there was no correlation found between IEG expression and the number of elements the juvenile copied from its tutor's song within the CM (Bolhuis et al. 2000; 2001; Terpstra et al. 2004).

Learning and Memory within the NCM and CM of Juvenile Females

Within the NCM of juvenile females, studies examining mechanisms of learning and memory suggest that this brain region is associated with song processing, but at a later developmental point in time than in juvenile males. Unlike in males, hearing conspecific song did not induce a *ZENK* response within the NCM of P30 females or result in an increase in pS6 within the NCM of P23 or P30 females (Bailey and Wade 2003, Ahmadiantehrani and London 2017). Song induced *ZENK* expression within the NCM was first identified in P45 females (Bailey and Wade 2005). However, there is electrophysiological evidence that individual neurons within the NCM show selectivity for conspecific song over heterospecific song as early as P20 and this selectivity is maintained at least until P30 (Stripling et al. 2001).

Within the CM of juvenile females, studies examining mechanisms of learning and memory suggest that this brain region may not function in the same way as juvenile female NCM. Hearing conspecific song did not induce a *ZENK* response within the CM of P30 or P45 females or result in an increase in pS6 within the NCM or CM of P23 or P30 females (Bailey and Wade 2003; 2005; Ahmadiantehrani and London 2017).

Overall, it is difficult to make definitive conclusions about the functions of NCM and CM in juvenile females in the context of song processing and sensory song learning due to the lack of comprehensive molecular and electrophysiological studies during the period for developmental song learning. The question remains: do juvenile males and females perform molecularly distinct forms of sensory song learning?

Chapter II: Experience-dependent Protein Synthesis in Juvenile Males and Females during Developmental Learning.

Introduction

Long-term stable memory formation requires that experience initiates new protein synthesis (Kandel and Spencer 1968; Gordon 1969; Glassman 1969; Uphouse et al. 1974; Klann et al. 2004; Davis and Squire 1984; Matthies 1989; Smalheiser et al. 2010; Griggs et al. 2013; Rajasethupathy et al. 2009; Litvin and Anokhin 2000; Ponomarenko and Kamyshev 1997; Ahmadiantehrani and London 2017; London et al. 2009). One molecular indication of new protein synthesis is the phosphorylation of the ribosomal protein S6(pS6) (Garza-Lombo and Gonsebatt 2016; Graber et al. 2013; Hay and Sonenberg 2004; Hoeffer and Klann 2010). pS6 has been used as a molecular indicator of learning and memory in a variety of tasks in vertebrates (Tang et al. 2002, Dash et al. 2006, Hoeffer et al. 2008, Chwang et al. 2007, Belelovsky 2009, Ahmadiantehrani and London 2017). We can therefore leverage what we know about pS6 to investigate how age, genetic sex, and experience influence developmental learning in zebra finch songbirds (*Taeniopygia guttata*).

Both male and female zebra finches form memories of song during development, but adult males learn to sing and females do not. We do not know if juvenile female sensory song learning is the same kind of sensory song learning as in juvenile males because females do not

sing. Both males and females have the necessary neural circuitry to support sensory song learning and the behavioral output of song preference. Thus, the sex-specific adult behaviors may arise from differences in which molecular and electrophysiological mechanisms of learning are utilized across sex in development. The brain region we used to investigate the mechanisms of juvenile sensory song learning in males and females during development is the auditory forebrain. The auditory forebrain is a brain region required for sensory song learning in juvenile males and is implicated in sensory song learning in juvenile females (London and Clayton 2008; Ahmadiantehrani and London 2017; Bailey and Wade 2005; 2003; Tomaszycki et al. 2006).

In juveniles, hearing song does not always affect molecular and electrophysiological signatures of learning within the auditory forebrain in the same way across sex or across age (Stripling et al. 2001; Bailey and Wade 2003; 2005; Tomaszycki et al. 2006; Ahmadiantehrani and London 2017). However, by adulthood, hearing song affects both the male and female electrophysiological and molecular signatures of learning within the auditory forebrain (Ahmadiantehrani et al. 2018; Chew et al. 1996; Scully et al. 2017). For example, it has been shown that at P30, hearing song increases pS6 in juvenile males and has no effect on pS6 in females, but in adulthood, hearing song increases pS6 in both males and females. This raises the question: What can pS6 tell us about sensory song learning ability in juvenile males and females between P30 and adulthood?

To answer this question, we used immunohistochemistry to measure the normalized pS6 cell density (pS6+) within the auditory forebrain of juvenile male and female zebra finches. Immunohistochemistry (IHC) is a technique used to determine the tissue distribution of the S6

protein (**Figure 2-1**). To evaluate the potential to learn from song, birds must be able to process song and generate a molecular response, so we compared birds following an acute song experience to those left in silence (**Figure 2-2)**. To test pS6+ during a known period of developmental learning in both sexes we selected ages; P30, P45, P60, and P67 (Eales 1985; 1987; Bohner 1983; 1990; Slater et al. 1991; Roper and Zann 2006; Chen et al. 2017; Miller 1979; Clayton 1988; Riebel 2000; 2003; Riebel et al. 2002; Lauay et al. 2004; Terpstra et al. 2004; Holveck and Riebel 2014; Chen et al. 2017; Diez et al. 2021) (**Figure 2-2)**. The accumulation of experience can influence developmental learning via closing a sensitive period), so to test the role of accumulated tutor song experience, independent of age, on the ability to form song memories we used two groups of P67 males: males who receive tutor song experience (Tutored) and those who have been isolated from tutor song (Isolate) (**Figure 2-2)**. Our results suggest that one molecular indicator of sensory song learning in males may be different from that of females during the sensory song learning period of development. More data about the electrophysiological and molecular mechanisms of learning and memory in males and females across the sensory song learning period of development would be necessary to be able to make more definitive conclusions about the sensory song learning abilities in males compared to females.

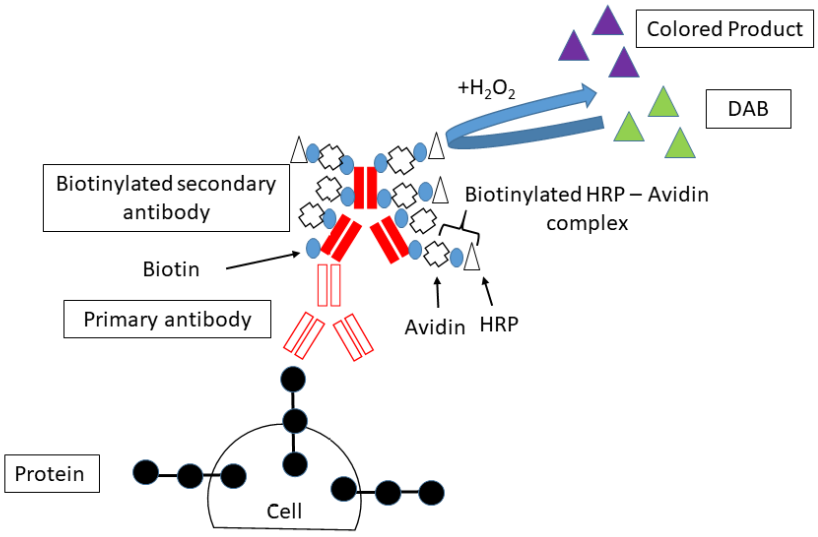

Figure 2-1 Diagram of immunohistochemistry (IHC) method.
IHC is a technique that uses specific antibodies to determine the tissue distribution of a specific protein. Primary antibodies (red outline) target specific proteins (black circles). A biotinylated secondary antibody (antibody is the solid red, Biotin is the small blue balls) can then bind to the primary antibody. The secondary antibody Fab domain binds to Fc domain of the primary antibody. The visualization method used for the IHC required a horseradish peroxidase (HRP) (small black triangles) mediated reaction. The way that HRP is added to the antibody-antibody complex is through the Avidin–Biotin Complex (ABC) method. Biotinylated horseradish peroxidase (HRP) is incubated with Avidin (the small black plus signs) and forms a Biotinylated HRP – Avidin complex. One Avidin molecule can bind four Biotin molecules. The ABC solution is incubated for an amount of time that allows for the incomplete saturation of Avidin with Biotin, so that when the ABC solution is added to the tissue, the ABC complex can then bind to the biotinylated secondary antibody. In an IHC in which 3,3'- Diaminobenzidin (DAB) is the visualization method, the HRP enzyme catalyzes the oxidation of DAB (green triangles). When hydrogen peroxide (H_2O_2) is present, HRP oxidizes the DAB producing a dark purple/brown color (purple triangles).

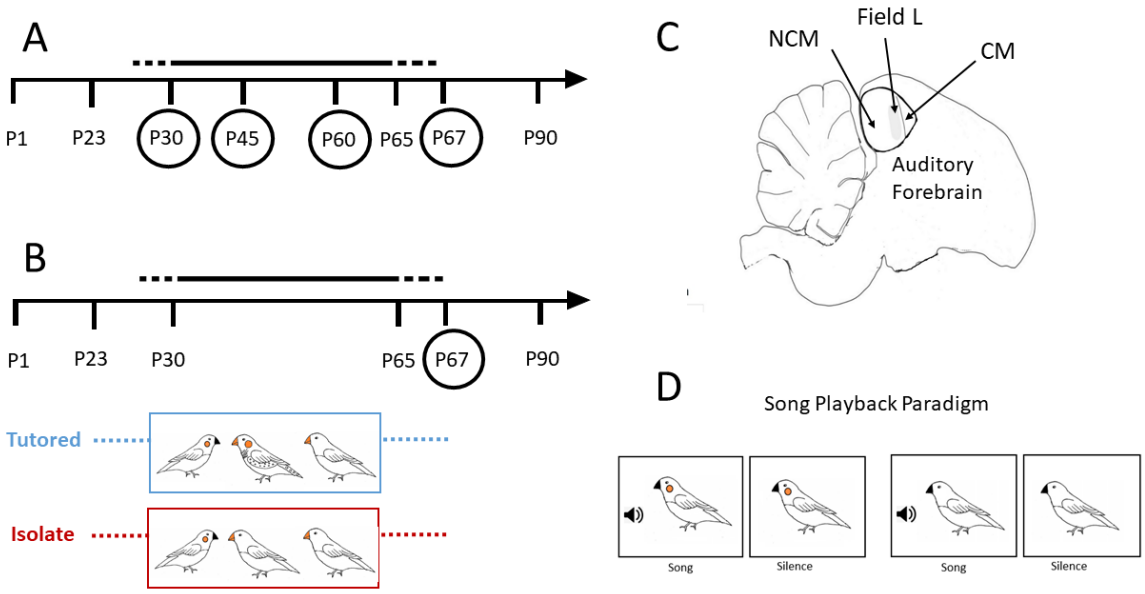

Figure 2-2 Experimental design.
Panel A depicts the ages of the "Normal" juvenile males and females which were collected. Six birds of each sex were collected at each age circled in black. Panel B depicts the ages of the "Tutored" and "Isolate" P67 males collected. Juvenile males (black beak with orange cheek patch) reared in the Tutored condition were reared with one adult male (orang beak with orange cheek patch) and one adult female (orange beak with no cheek patch). Males reared in the Isolate condition were reared with two adult females. Males were removed from the aviary at P23 and placed in temporary cages with other P23 juveniles and adult females until the males reached P30. At P30, the males were moved into their respective conditions. They remained in these conditions until P66 then were placed into soundproof chambers to be collected the morning of P67. The Tutored condition is outlined in blue. The dashed line begins at P23, the age at which the rearing condition for the Tutored birds becomes different than Normal birds, includes a depiction of the different rearing environment, and concludes at P67 when these birds are collected. The Isolate condition, outlined in red, to depicts the same things. Panel C shows a depiction of the neuroanatomical location of the auditory forebrain and its sub-regions; NCM, CM, and Field L, within the zebra finch brain, adapted from Figure 3 in London 2019. Panel D shows a depiction of the soundproof chambers the birds were placed in on the night before the bird was collected.

Methods

Subjects.

All procedures were conducted in accordance with the NIH guidelines for the care and use of animals for experimentation and were approved by the University of Chicago Institutional Animal Care and Use Committee (ACUP no. 72220).

Rearing.

All zebra finches were housed on a 14 hr:10 hr light:dark cycle, with seed and water provided *ad libitum*. The juveniles used in this study hatched in flight aviaries, amongst males and females of all ages. The "Normal" juvenile males and females, 24 per sex, were raised in a breeding aviary and were tutored under normal social conditions (i.e., by their father or other adult males in the breeding colony). The "Tutor" and "Isolate" experimental males, 6 per condition, were removed from the aviaries and placed in a sound attenuated chambers at P23 with one to two other juveniles and two foster females. At P30, all of the males were moved. The "Tutor" males were placed in a new chamber with one tutor male and a companion adult female and the "Isolate" males were placed in a chamber with two new companion adult females. All birds remained in their respective rearing conditions until the night prior to collection, at which point the birds were moved into individual acoustic chambers 3 hours before lights off to facilitate the song playback paradigm on the morning of collection.

Song Playback Paradigm.

All experimental birds were either exposed to a song playback or left in silence. All birds were placed into a soundproof chamber overnight on the evening before they were to be

collected. The following morning, two hours after lights come on, those in the song group heard 30 min of a song not heard previously ("Song" condition). Age-, sex-, and rearing condition-matched controls heard no song playback ("Silence" condition). There were three pairs of Song and Silence condition, n = 3, birds for all age- sex- and rearing condition- combinations (Mello et al. 1992). Birds in the Song condition were exposed to novel conspecific song, "zf101". This song was obtained from a bird bred and raised more than 20 years ago at the Ohio State University by Dr. Susan Volman and were thus novel to all subjects. The acoustic stimuli were played back through a speaker (model FE103, RadioShack) located in the chamber. The average sound intensity was adjusted to 70 dB via a sound pressure meter (model 33-2055, RadioShack).

Immunohistochemistry.

Immediately after hearing a song playback or being left in silence, the birds were anesthetized with Isoflurane and were transcardially perfused with ice-cold 0.1M PBS, followed by 4% paraformaldehyde in 0.025M PBS. Brains were dissected and post-fixed overnight at 4°C. They were then embedded in gelatin (8% in 0.1M PBS) and fixed overnight at 4°C. Gelatin embedded brains were cryoprotected first in 15% and then 30% sucrose in 0.1M PBS. Brains were sectioned into 50µm sagittal sections on a Thermo NX50 Cryostat, in a series of six into a solution of 0.1M PBS and 0.01% Sodium Azide to protect from fungal growth.
We performed immunohistochemistry with all sections from a single series from midline to ~990µm lateral for each bird to capture the extent of auditory forebrain.

Sections were initially washed three times in 0.01M PBS for 10 mins each to clear the Sodium Azide from the tissue. After permeabilization with 0.3% Triton-X in 0.1M PBS (30

min), endogenous peroxidases were exhausted with 2% H2O2 in 0.1M PBS containing 0.05% Tween-20 (PBST) for 15 min. After extensive washes in PBST, sections were blocked with 3% normal serum for 60 min at room temperature. Sections were incubated with primary antibodies overnight at 4°C, followed by PBST washes and a 60 min room-temperature incubation with biotinylated secondary antibodies. After washing with PBST, sections were incubated in avidin-biotin complex (Vectastain Elite ABC Kit; Vector Laboratories) for 30 min at room temperature. The peroxidase complex was visualized with 3,3′-Diaminobenzidine (DAB, Sigma, St. Louis, MO, USA) containing 0.003% H2O2 in 0.1M PBS. Sections were then mounted, dehydrated, cleared, and coverslipped with Permount (Fisher Scientific).

Antibodies and serum were as follows. pS6: rabbit anti-pS6 primary antibody (1:500 in 1% NGSPBST; Cell Signaling Technologies #2211) blocked in normal goat serum (NGS), with a biotinylated goat anti-rabbit IgG secondary (1:500; Vector Laboratories); S6: goat anti-S6 primary antibody (1:2000 in 1% NHS-PBST; Santa Cruz Biotechnology) blocked in normal goat serum (NGS), with a goat anti-mouse IgG secondary antibody (1:500; Vector Laboratories).

Image Acquisition and Processing.

All images were captured using a Zeiss Axiovert 200M microscope with a Zeiss Axiocam digital color CCD camera (Carl Zeiss Microscopy, Thornwood, NY, USA) running Slidebook 5.5 software (Intelligent Imaging Innovations, Denver, CO, USA). Each Superfrost Plus glass microscope slide held 5 sections spanning the medial-lateral extent of the auditory forebrain in each bird. For all brain sections, we obtained images that contained all three sub-regions of the auditory forebrain; Field L, NCM, and CM, plus the adjacent hippocampus (HP)

with a 5X objective. Neuroanatomical landmarks were identified using the Histological Atlas on the Zebra Finch Expression Brain Atlas (ZEBrA, Oregon Health and Science University, Portland, OR, USA: zebrafinchatlas.org). These landmarks were visible using the 5X objective and used to quantify NCM, CM, and HP across brain sections and individuals. The landmarks for the NCM are as follows; dorsal boundary landmarks = hippocampus, area parahippocampalis (APH), and lateral ventricle, posterior boundary landmarks = cerebellum and posterior telencephalon, ventral boundary landmarks = choroid plexus (ChP) and septum, anterior boundary landmarks = Field L. The landmarks for the CM are as follows; dorsal and anterior boundary landmarks = area parahippocampalis (APH), posterior boundary landmarks = Field L, ventral boundary landmarks = ChP, nidopallium. The landmarks for the Field L are as follows; dorsal, posterior, and ventral boundary landmarks = NCM, anterior boundary landmarks = CM.

Each image was opened in ImageJ and the image was then transformed into an 8-bit image. The region of interest was manually circled with the freehand selection tool and the threshold was adjusted to most accurately highlight the cells within the region of interest. The area, standard deviation, integrated density, limit to threshold, mean gray value, and modal gray value were all measured and recorded. Using the invert table - a table where you can invert the color of the image without changing the pixel value - the image was then flipped via “Invert LUT”. This was done in order for the ImageJ algorithm to easily distinguish cells from background, and the number of cells were recorded in pixels^2 with the minimum size requirement of 3 pixels^2. All measures were corrected for the size of the measured area. The number of labeled cells within the HP was quantified to confirm that the staining within does not change systematically with sex, age, or song exposure before using it as technical control. The number

of labeled cells was quantified within the NCM and CM and was normalized to the values from the adjacent hippocampus (HP), using ImageJ (Mello and Clayton 1994). Also, a random number generator was used to determine the order of images to be analyzed in order to minimize the potential for systematic human error.

Statistics.

We analyzed normalized NCM and CM values from the IHC with two- and three-way ANOVAs (SPSS; $\alpha = 0.05$) to test for main effects of age, acute song experience, rearing condition, and sex, and their corresponding interactions. Three-way ANOVAs were performed on all normalized values from the NCM and CM from "Normal" birds and two-way ANOVAs were performed on all normalized values from the NCM and CM from the P67 male groups: Normal, Tutored, and Isolate.

Results

No differences in the normalized S6 cell density (S6+) or normalized pS6 cell density (pS6+) values within the HP across age, sex or acute song experience.

We first confirmed that neither S6+ nor pS6+ in the HP changed across experimental conditions. We did this so that the S6+ and pS6+ values from the NCM and CM could be normalized to the HP values on each section, which allows us to control for potential technical differences in staining intensity across batches or from non-specific background staining.

A three-way ANOVA was conducted to examine the effect of age, sex and acute song experience on the S6+ in the HP of both Normal juvenile males and females in response to hearing song and when left in silence. There were no significant main effects of age ($F_{(1,40)}$ = 2.23, p = 0.14), sex ($F_{(1,40)}$ = 0.82, p = 0.37), or acute song experience ($F_{(1,40)}$ = 0.60, p = 0.44) (**Figure 2-3**). For the two- and three-way interactions, we found no significant interaction between age and sex ($F_{(1,40)}$ = 0.41, p = 0.53), age and acute song experience ($F_{(1,40)}$ = 0.1.40, p = 0.24), sex and acute song experience ($F_{(1,40)}$ = 0.12, p = 0.73) or the three-way interaction between age, sex and acute song experience ($F_{(1,40)}$ = 0.57, p = 0.45). A two-way ANOVA was conducted to examine the effect of rearing condition and acute song experience on the S6+ cell densities in the HP of the P67 male groups response to hearing song and when left in silence. There were no significant main effects of rearing condition ($F_{(2,12)}$ = 1.25, p = 0.32) or acute song experience ($F_{(1,12)}$ = 1.64, p = 0.22) (**Figure 2-4**). For the two-way interaction, we found no significant interaction between rearing condition and acute song experience ($F_{(2,12)}$ = 0.08, p = 0.93).

No differences in the pS6+/S6+ cell density of birds in the Silence condition across age and sex.

To confirm there was no difference in pS6+/S6+ cell density at baseline in the NCM and CM, a two-way ANOVA was conducted to examine if there is an effect of age or sex on the pS6+/S6+ cell density in NCM and CM of Normal juvenile males and females when left in silence. Within the NCM, there was no significant main effect of age ($F_{(3,22)}$ = 1.09, p = 0.38), sex ($F_{(1,22)}$ = 0.10, p = 0.76), or the interaction between the two ($F_{(3,22)}$ = 2.03, p = 0.14) (**Figure 2-3**). Within the CM, there was no significant main effect of age ($F_{(3,22)}$ = 0.49, p = 0.69), sex

($F_{(1,22)} = 2.06$, $p = 0.17$), or the interaction between the two ($F_{(3,22)} = 0.55$, $p = 0.65$) (**Figure 2-3**). A one-way ANOVA was conducted to examine if there is an effect of rearing condition on the pS6+/S6+ cell density in NCM and CM of the P67 male groups. Within the NCM, there was not a significant effect of rearing condition ($F_{(2,6)} = 2.28$, $p = 0.18$) (**Figure 2-4**). Within the CM, there was not a significant effect of rearing condition ($F_{(2,6)} = 1.63$, $p = 0.27$) (**Figure 2-4**).

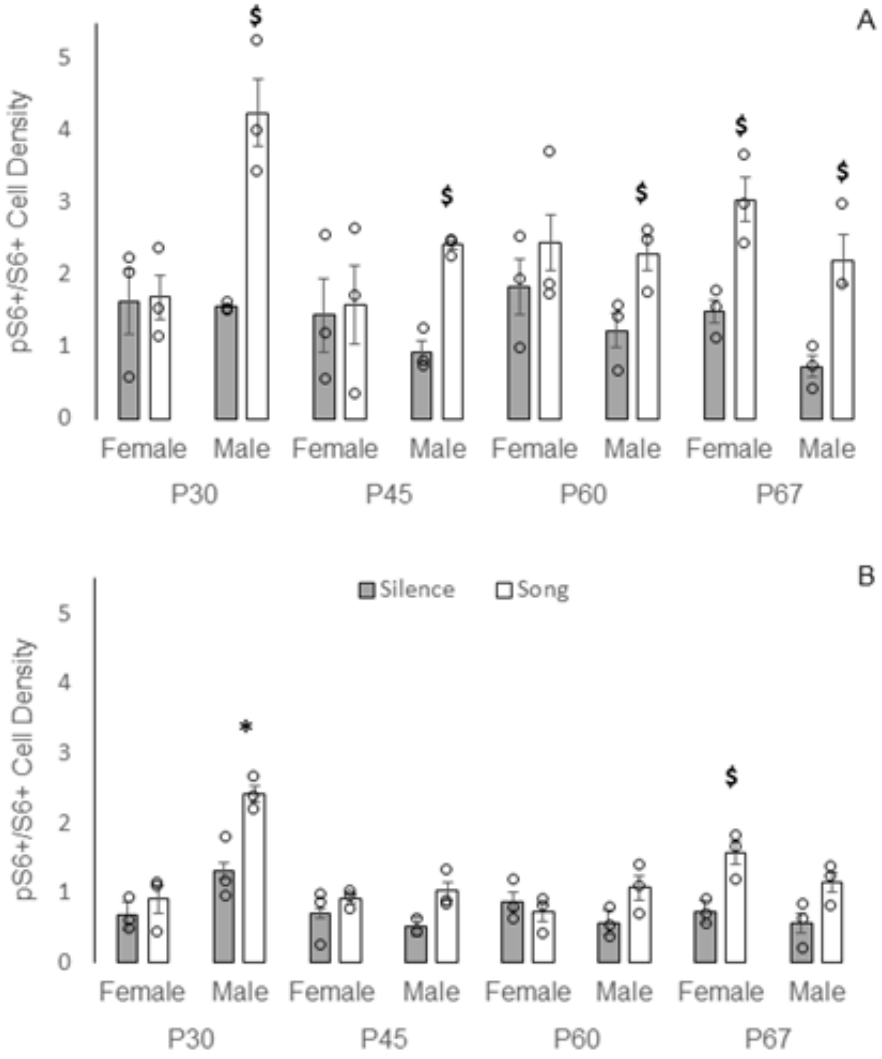

Figure 2-3. The effects of acute song exposure on the pS6+/S6+ cell density within CM and NCM of Normal males and females.

The pS6+/S6+ cell density in CM (A) and NCM (B) from both male and female Song and Silence Normal birds across all ages are shown. Bars (white, Song; gray, Silence) represent the experimental group mean ± SEM. Open circles (○) denote individual birds. $$P < 0.05$ across Song and Silence P67 males and females. *$P < 0.05$ between all other age- sex- and acute song experience- matched birds.

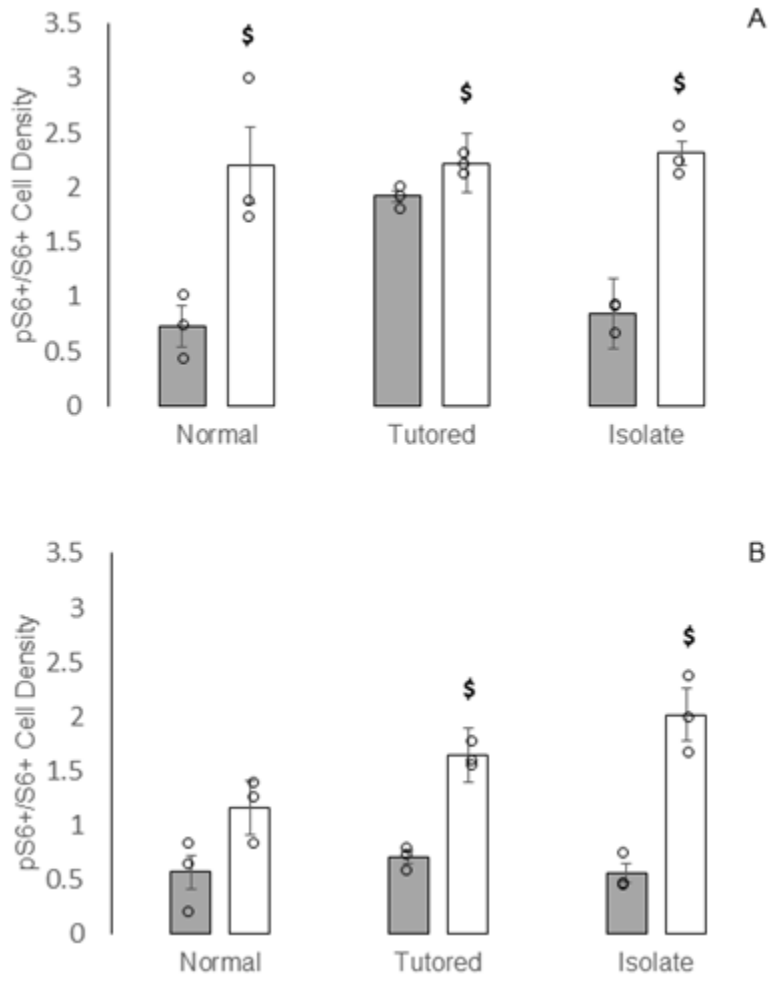

Figure 2-4. The effects of acute song exposure on the pS6+/S6+ cell density within CM and NCM of P67 males raised with different numbers of adult tutor males.
The pS6+/S6+ cell density in CM (A) and NCM (B) from the P67 male Song and Silence birds from each rearing condition. Bars (white, Song; gray, Silence) represent the experimental group mean ± SEM. Open circles (○) denote individual birds. $\$P < 0.05$ across Song and Silence P67 males from each condition.

In NCM of Normal birds, sex and age influence the effect of hearing song playbacks

A three-way ANOVA was conducted to test if there are effects of age, sex and acute song exposure on the pS6+/S6+ cell density in the NCM of the Normal birds. We found significant main effects of age ($F_{(3,35)} = 6.94$, $p < 0.01$), sex ($F_{(1,35)} = 9.17$, $p < 0.01$) and acute song experience ($F_{(3,35)} = 10.27$, $p < 0.01$) (**Figure 2-3**). Post-hoc Tukey's HSD analysis of the age data showed that the P30 NCM is significantly different from all other ages (P30-P45: $p < 0.01$ P30-P60: $p = 0.01$, P30-P67: $p < 0.01$) (**Table S1**). Post-hoc Tukey's HSD analysis for sex showed that males were significantly different than females ($p < 0.01$) (**Table S1**). Post-hoc Tukey's HSD analysis for acute song experience showed that the NCM of birds in the Song condition were significantly different than the NCM of birds in the Silence condition ($p < 0.01$) (**Table S1**). For the two- and three-way interactions, we found a significant interaction between age and sex ($F_{(3,35)} = 6.48$, $p < 0.01$) and between sex and acute song experience ($F_{(1, 35)} = 9.01$, $p < 0.01$), but not between age and acute song experience ($F_{(3,35)} = 2.23$, $p = 0.10$) or the three-way interaction between age, sex and rearing condition ($F_{(3,35)} = 0.89$, $p = 0.45$) (**Table S1**). Post-hoc Tukey's HSD analysis for age by sex showed that the NCM of P30 males were significantly different than the NCM of females at all ages (P30 Male-P30 Female: $p < 0.01$, P30 Male-P45 Female: $p < 0.01$, P30 Male-P60 Female: $p < 0.01$, P30 Male-P67 Female: $p < 0.01$) and compared to the NCM of males at all other ages (P30 Male-P45 Male: $p < 0.01$, P30 Male-P60 Male: $p < 0.01$, P30 Male-P67 Male: $p < 0.01$) (**Table S1**). Post-hoc Tukey's HSD analysis for sex by acute song exposure showed that within the NCM of males hat heard song playbacks were significantly different than males left in silence, or females that either heard song playbacks or

were left in silence (Male Song-Male Sil: $p < 0.01$, Male Song-Female Song: $p < 0.01$, Male Song-Female Sil: $p < 0.01$) (**Table S1**).

There is a main effect of hearing song playbacks which is not dependent on age and sex, within the CM of Normal birds.

A three-way ANOVA was conducted to test if there are effects of age, sex and acute song exposure on the pS6+/S6+ cell density in the CM of the Normal birds. We found a significant main effect of acute song exposure ($F_{(1,35)} = 10.77$, $p < 0.01$), but not of age ($F_{(3,35)} = 2.78$, $p = 0.06$) or sex ($F_{(1,35)} = 1.81$, $p = 0.19$) (**Figure 2-3**). Post-hoc Tukey's HSD analysis for acute song experience showed that the CM of birds in the Song condition were significantly different than the CM of birds in the Silence condition ($p < 0.01$) (**Table S2**). For the two- and three-way interactions, we found a significant interaction between age and sex ($F_{(3,35)} = 3.17$, $p = 0.04$), and between sex and acute song experience ($F_{(1, 35)} = 11.36$, $p < 0.01$), but not between age and acute song experience ($F_{(3,35)} = 0.80$, $p = 0.50$) or the three-way interaction between age, sex and rearing condition ($F_{(3,35)} = 0.49$, $p = 0.69$) (**Table S2**). Post-hoc Tukey's HSD analysis for age by sex showed that the CM of P30 males were significantly different than the CM of P30 females, P67 females and P67 males (P30 Male-P30 Female: $p = 0.05$, P30 Male-P67 Female: $p = 0.02$, P30 Male-P67 Male: $p = 0.02$) (**Table S2**). Post-hoc Tukey's HSD analysis for sex by acute song exposure showed that the CM of males that heard song were significantly different than the CM of males left in silence and the CM of females that either heard song or were left in silence (Male Song-Male Sil: $p < 0.01$, Male Song-Female Song: $p = 0.01$, Male Song-Female Sil: $p = 0.01$) (**Table S2**).

In P67 male NCM, a significant increase in pS6+/S6+ cell density after acute song playback experience depends on rearing condition.

To investigate the influence of accumulated song experience on pS6+/S6+ cell density in the NCM independent of age, a two-way ANOVA was conducted. We compared the pS6+/S6+ cell density in the Silence and Song conditions from the NCM of the P67 Normal, Tutored and Isolate males. The two-way ANOVA showed a significant main effect of acute song experience ($F_{(1,12)} = 9.87$, $p < 0.01$), but no significant main effect of rearing condition ($F_{(2,12)} = 1.18$, $p = 0.34$) (**Figure 2-4**). Additionally, there was a significant interaction between acute song experience and rearing condition ($F_{(2,12)} = 7.51$, $p < 0.01$) (**Table S3**). Post-hoc Tukey's HSD analysis showed that song playbacks resulted in a significant difference in the density of pS6+/S6+in Isolate NCM that heard song playback compared to three groups: the NCM of Isolate birds left in silence ($p < 0.01$), the NCM of Normal birds left in silence ($p = 0.01$), and the NCM of Tutored birds that heard song playback ($p = 0.04$) (**Table S3**). The pS6+/S6+ cell density within the NCM of Isolate birds that heard song was greater than the pS6+/S6+ cell density within NCM of Tutor birds that heard song was greater than the pS6+/S6+ cell density within the NCM of Normal birds left in silence was greater than the pS6+/S6+ cell density within the NCM of Isolate birds left in silence.

In P67 male CM, the significant increase in pS6+/S6+ cell density after acute song playback experience is not significantly altered by rearing condition.

To investigate the influence of accumulated song experience on pS6+/S6+ in the CM independent of age, a two-way ANOVA was conducted. We compared the pS6+/S6+ cell density in the Silence and Song conditions from the CM of the P67 Normal, Tutored and Isolate males. Two-way ANOVA showed a significant main effect of acute song experience ($F_{(1,12)} = 4.77$, $p = 0.05$), but no significant main effect of rearing condition ($F_{(2,12)} = 0.01$, $p = 0.99$) (**Figure 2-4**). Additionally, there was no significant interaction between acute song experience and rearing condition ($F_{(2,12)} = 3.01$, $p = 0.09$) (**Table S4**).

Discussion

The goal of this study was to further understand the differences in the pS6 song response across maturation, sex, and accumulated song experience between P30 and adulthood. In juveniles, there is a distinct difference in the pS6+/S6+ song response between sexes, but in adulthood, both males and females show an increase in pS6+/S6+ response to hearing song playbacks. Our results suggest that females process hearing song playbacks in the same way as males – hearing song does not activate a song responsive molecular mechanism that could indicate song learning is occurring between P30 and P67 or that the pS6 mechanism of sensory song learning does not contribute to facilitating sensory song learning in females until P67. Also, our results suggest that at least one mechanism of sensory song learning within the auditory forebrain of males may be different from that of females between P30 and P67.

As expected, based on the fact that an increase in the density of pS6+/S6+ has been reported in P30 and adult male CM after song playbacks, we find that all ages we examined in between also show a song response within the CM (Ahmadiantehrani et al. 2018;

Ahmadiantehrani and London 2017) (**Figure 2-3**). Unlike in CM, we detected a significant song response in P30 males NCM but not at any other age (**Figure 2-3**). This is surprising; the song response seen in P30 males from our study replicates prior pS6 results and there is a significant pS6 song response within the NCM of adult males (Ahmadiantehrani et al. 2018; Ahmadiantehrani and London 2017). Once hearing song playbacks elicits a molecular response at P30, it is unlikely that song experience becomes no longer salient between P30 and adulthood, especially given that we know males use NCM to perform tutor song memorization between P30 and P65 (Ahmadiantehrani and London 2017; London and Clayton 2008; Gobes and Bolhuis 2007, Phan et al. 2006; Adret et al. 2012; Yanagihara and Yazaki-Sugiyama 2016).

The difference in pS6+/S6+ cell density between CM and NCM is inconsistent with a different molecular mechanism of learning and memory, immediate early genes (IEG), and electrophysiological findings. Studies investigating the IEG *ZENK* (*zif268, egr-1, ngfi-a, krox24*) have found that acute song experience results in an induction of *ZENK* between P30 and P60 in both the CM and NCM (Bailey and Wade 2003; 2005; Tomaszycki et al. 2006; Jin and Clayton 1997; Gobes et al. 2010). However, not all IEGs are expressed at the same ages or are induced as a result of acute song experience within the CM and NCM (Bailey and Wade 2005). Electrophysiological data investigating both individual and groups of neurons within juvenile male NCM suggest that the neural response to song is selective for specific songs during the sensory song learning period (Stripling et al. 2001; Miller-Sims and Bottjer 2014; Yanagihara and Yazaki-Sugiyama 2016). Neurons within the NCM show selectivity not only for conspecific song, but for tutor song between P30 to P60 (Stripling et al. 2001; Miller-Sims and Bottjer 2014; Yanagihara and Yazaki-Sugiyama 2016). The neurons which were recorded from were recorded

from more lateral portions of the NCM compared the regions of the NCM which were measured for *ZENK* induction – electrophysiological recordings were recorded from cells roughly 0.2 mm or greater lateral. In order to be able to thoroughly compared electrophysiological data to molecular data within the NCM, a cell type analysis should be done. This analysis should analyze which types of cells were recorded from in electrophysiological studies and the proportion of this cell type should be determined within the area measured for *ZENK* induction in order to make conclusions about if the neurons showing song selectivity are the same as the neurons which show *ZENK* induction. This kind of comparison study has not yet, to our knowledge, been done performed

When comparing across the three groups of P67 males, we detected a significant song response within the NCM and CM of the P67 Tutored and P67 Isolate males (**Figure 2-4**). In the P67 Normal males, we only detected a significant song response in CM - the pS6+/S6+ fold-difference between the Song and Silence conditions is 3-fold in NCM but falls shy of being statistically significant (**Figure 2-4**). These results are surprising because song playbacks produce a robust pS6+/S6+ response in adult males and functional imaging in P67 males indicates no difference in the global functional connectivity strength of neural networks within the NCM of P67 Tutored and P67 Normal male NCM (Ahmadiantehrani et al. 2018; Layden et al. 2020). Detection of a song response reflects both the pS6+/S6+ levels after acute song playbacks, and those at baseline (Silence condition) so it is possible that differing levels of pS6 in the Silence condition birds affected these outcomes. In P67 Normal males, the density of pS6+/S6+ of birds in the Silence condition is significantly greater in the CM than the NCM. Within the CM, the density of pS6+/S6+ of birds in the Silence condition is significantly higher

in P67 Tutored than in the P67 Isolate or P67 Normal. These data confirm that two different types of learning, tutor song memorization and adult song recognition learning, do not necessarily have distinct patterns of S6 phosphorylation following song playback experience. This implies that the accumulation of song experience may not influence the pS6+/S6+ cell density measurement of developmental learning.

Even though we know females form lasting memories of song between P30 and P67, we did not detect a significant pS6+/S6+ song response in NCM or CM until P67 (**Figure 2-3**) (Riebel 2000; Miller 1979; Riebel et al. 2002; Riebel 2003; Clayton 1988; Clayton 1990; Braaten and Reynolds 1999; Lauay et al. 2004). If there is sensory song learning happening between P30 and P67, then we would expect to see a significant increase in pS6+/S6+ cell density in response to hearing song playbacks compared to Silence birds. One possible interpretation of our findings is that females have established song preference by P30. We do not know the exact age at which Normal females establish song preference, but we do that preference is established between P25 and P35 (Clayton 1988; Miller 1979; Riebel 2000). The hypothesis that females have established preference by P30 would also be consistent with prior data showing the lack of a pS6+/S6+ song response within the NCM and CM at P30 females (Ahmadiantehrani and London 2017). If females do establish preference by P30, then our data would suggest that females do not learn again until P67. The pS6 song response at P67 could indicate the beginning of adult song recognition learning. This is consistent with prior data that show a pS6+/S6+ song response in the CM and NCM of adult females (Ahmadiantehrani et al. 2018). It is not unreasonable to suggest that once females have established song preferences they do not do any more sensory song learning until adult song recognition learning, because we know that we cannot overwrite

female song preference once it has been established (Riebel 2000). However, our data are not consistent with existing IEG and electrophysiological data. These data suggest that the electrophysiological response to song appears by P20 and that the molecular song response appears sometime between P30 – P45 (Bailey and Wade 2003; 2005; Tomaszycki et al. 2006; Stripling et al. 2001). One potential explanation is that IEG expression and pS6 develop on distinct timescales. Another explanation is that preference may already be established, so there is no response due to no additional learning and memory. Alternatively, the NCM and CM may not be necessary for juvenile female sensory song learning, however this is unlikely given the existing IEG and ancestral behavioral data (Bailey and Wade 2003; 2005; Tomaszycki et al. 2006; Stripling et al. 2001; Odom et al. 2014; Choe et al. 2021).

The function of the CM and NCM in juvenile sensory song learning may not be the same within sex or across sex. When comparing CM to NCM in males, the results from this study support that these brain regions may not have the same function in juvenile male sensory song learning. This is consistent with prior molecular data: hearing song induces IEGs at P30, P45, P60 in NCM, but only at P30 in CM (Bailey and Wade 2003; 2005; Tomaszycki et al. 2006; Ahmadiantehrani et al. 2018). When comparing CM to NCM in females, the results from this study suggest that these brain regions may not have a function in sensory song learning. However, the phosphorylation of S6 is just one metric of learning and this does not exclude the possibility that other mechanisms of learning and memory are operating within these brain regions during this period of development. The lack of a significant pS6+/S6+ song response does not necessarily mean that the bird is not learning. It is unknown what proportion of cells within the auditory forebrain are needed to have experience-dependent new protein synthesis to

support learning. It would be unlikely that no cells within the auditory forebrain have pS6 and the bird is still learning, because the ribosome must be active for protein synthesis to occur. In order to determine what proportion of cells within the auditory forebrain is needed to have experience-dependent new protein synthesis, one would need to be able to selectively inhibit the phosphorylation of S6 in individual cells. In trying to compare the function of the CM and NCM across sex in the context of juvenile sensory song learning, the data from this study suggest that pS6 in male CM and NCM is a marker of sensory song learning ability, and the function of these two sub-regions may not be the same in males compared to females during sensory song learning. However, more information is needed about both of these sub-regions in females to make reasonable conclusions about similarities and differences in function within females and across sex.

There are possible technical and biological explanations for the discrepancies between our results and prior molecular data measuring ZENK in response to acute song exposure within the NCM and CM (Bailey and Wade 2003, Bailey and Wade 2005, Tomaszycki et al. 2006). One technical explanation could come from differences in image processing. In this study, we needed to stitch together multiple images to enable comprehensive quantification of NCM and CM, thus creating uneven illumination along image edges which could have contributed to variation in cell density counts. Uneven illumination around the edges of the images makes it difficult to alter the threshold of the image in a way that accurately highlights all of the stained cells when trying to calculate cell density. The proportion of cells which were captured by adjusting the threshold was maintained across all images to the best of my ability. An alternative strategy that others have used, which may have been chosen to avoid the issue of stitching together images, is to

capture single images within these larger sub-regions (Bailey and Wade 2003; 2005; Tomaszycki et al. 2006). For example, IEG proteins ZENK and c-FOS, when measured via single images captured from within the NCM, do show a statistically significant increase after hearing novel song in P45 males (Bailey and Wade 2005; Tomaszycki et al. 2006). We chose the more holistic measures because there are multiple cell subtypes distributed in pockets of the auditory forebrain and structural and functional neuroanatomy indicates that these regions are highly interconnected and work together (Bauer et al. 2008; Vates et al. 1996; Theunissen et al. 2004).

Conclusions

These results from this study suggest that one indicator of sensory song learning within the auditory forebrain of males may be different from that of females between P30 and P67. However, we are unable to make definitive statements about learning in juvenile males and females during this period of development: we do not know how sensory song learning is biologically facilitated in females, nor the specific ages at which females are capable of sensory song learning. More comprehensive studies exploring other molecular markers of learning in females at these ages would need to be done in order to make more definitive conclusions.

Chapter III: Exploring the Abundance of microRNA in Juvenile Males and Females during Developmental Learning Using *in situ* Hybridization (ISH)

Introduction

microRNA (miR) biogenesis

miRs can be located within the genome in a variety of places. miRs can be found within intergenic regions, within the introns of protein coding and non-coding genes, and within the exons of non-coding genes (Olena and Patton 2014; Kellis et al. 2014; Pasquinelli et al. 2000; Lee et al. 2002; Lau et al. 2001; Lagos-Quintana et al. 2001, 2003). Intergenic and intronic miRs can be monocistronic with their own promoters, or polycistronic, where several miRs are transcribed as a cluster of primary transcripts with a shared promoter. (Lee et al. 2002; Lau et al. 2001; Lagos-Quintana et al. 2001, 2003). Exonic miRs are rarer than the other types of miR and often overlap an exon and an intron of a non-coding gene (Rodriguez et al. 2004).

The biogenesis of miRs is conducted by two enzymes, Drosha and Dicer, which catalyze two subsequent processing events in the nucleus and cytoplasm (Michlewski and Caceres 2019). In the nucleus, genomic DNA is transcribed to generate a long primary miR (pri-miR), about 1000 nucleotides (nt) long, which is then cleaved by Drosha to form a precursor miR (pre-miR) with hairpin structure, about 70 nt long (**Figure 3-1).** These hairpin RNAs are transported to the

cytoplasm by nuclear export receptor exportin-5 and are cleaved by Dicer in the cytoplasm to form mature miRs (**Figure 3-1).** Mature miR are characterized as single-stranded RNA molecules that are approximately 21-23 nucleotides in length, have a uridine nucleotide at their 5'-end, and are partially complementary to one or more mRNA. Dicer cleavage can potentially produce two mature miR; a miR from the 5'-end (-5p) strand and one from the 3'- end (-3p) strand of the pre-miR (**Figure 3-1)**. A nucleic acid strand is inherently directional, the -5p end has a free hydroxyl (or phosphate) on a 5' carbon and the -3p end has a free hydroxyl (or phosphate) on a 3' carbon. Thus, -5p mature miR produced from the Dicer cleavage is the strand which has the free hydroxyl (or phosphate) on a 5' carbon and the -3p mature miR has free hydroxyl (or phosphate) on a 3' carbon. Mature single-stranded miR bind to the 3′-UTR region of the target mRNA, preventing the translation of the bound mRNA or by marking the target mRNA for degradation (O'Brien et al. 2018). Each miR, miR-5p and miR-3p, can be functional – i.e., can be independently regulated and can have multiple mRNA targets.

miR in Learning and Memory

miR play a critical role in learning and memory by regulating which – and how many of each – mRNA can be translated into protein (**Figure 3-2)** (O'Brien et al. 2018). Many miR are expressed in the brain and play important roles in facilitating long-lasting forms of synaptic plasticity that underlie learning and memory formation, such as miR-124 (Rajasethupathy et al. 2009, Yang et al. 2012, Wang et al. 2012; Wang et al. 2021; Aksoy-Aksel et al. 2014; Hu and Li 2017; Thomas et al. 2018). Reducing miR-124 levels through the infusion of a locked nucleic acids (LNA)-probe in the hippocampus of mice was found to restore spatial memory and social

interaction in adult mice, whereas AAV-mediated overexpression of miR-124 in the hippocampus impaired long-term potentiation and spatial memory in mice by regulating the expression of *ZENK*, demonstrating the prominent role of miR-124 in the negative regulation of synaptic plasticity and memory formation (Yang et al. 2012). Furthermore**,** miRs are differentially expressed throughout the brain during development and function in regulating sex-specific biological and behavioral phenotypes (Benoit et al. 2015; Mellios et al. 2011; Murphy et al. 2014; Sempere et al. 2004; Kapsimali et al. 2007; Choi et al. 2008; Gunaratne et al. 2011; Pfau et al. 2016; Hao and Waxman 2018; Gao et al. 2019). For example, we can look at the change in the miR expression profile within the Nucleus Accumbens (NAc), before and after Subchronic Variable Stress (SCVS), a stress paradigm that produces depression-like behavior in female mice, but not in males (Hodes et al. 2015). The NAc is a brain region essential for reward and emotion processing as well as the transcriptional and epigenetic processes within the NAc that have been linked to sex-specific stress responses (Westenbroek et al. 2003; Dalla et al. 2008; LaPlant et al. 2009; Sachs et al. 2014; Hodes et al. 2015). A previous study investigated miR expression profiles before and after SCVS within the NAc as a potential mechanism contributing to marked sex differences in stress-induced transcriptional patterns that may underlie heightened female stress susceptibility (Pfau et al. 2016). Researchers found that male and female mice exhibit largely non-overlapping miR profiles and enriched functional processes following SCVS, which contributes to the difference in the behavioral stress response between the sexes (Pfau et al. 2016). Thus, miR can be used as a tool when investigating the relationship between sex differences, maturation and learned behavior.

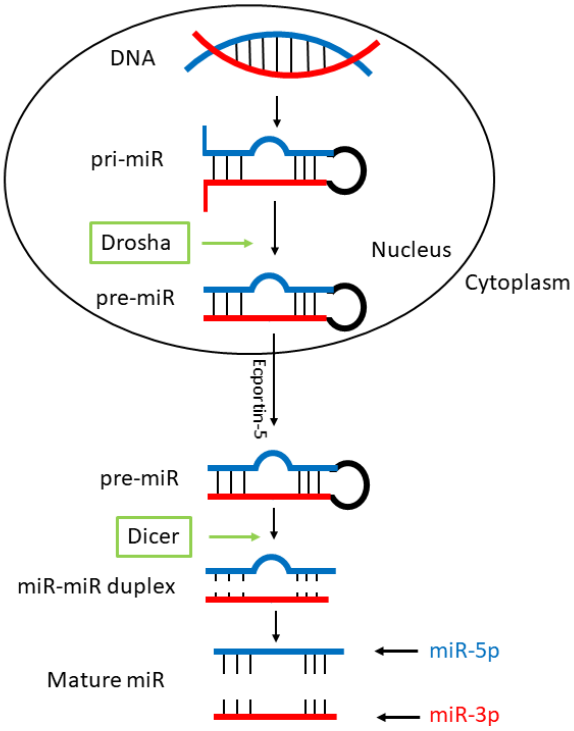

Figure 3-1 miR biogenesis.
When a miR gene is transcribed within the nucleus it forms pri-miR. The Drosha enzyme then cleaves off the nucleic acids that are not within the hairpin stem loop, which results in pre-miR. pre-miR gets transported from the nucleus into the cytoplasm by the Exportin-5 protein. The Dicer enzyme then catalyzes the degradation of the pre-miR into smaller components, the miR-miR duplex. The expression of one miR gene can result in two functional mature miRs, one from each strand; a miR-5p (indicated by the blue strand) and a miR-3p (indicated by the red strand).

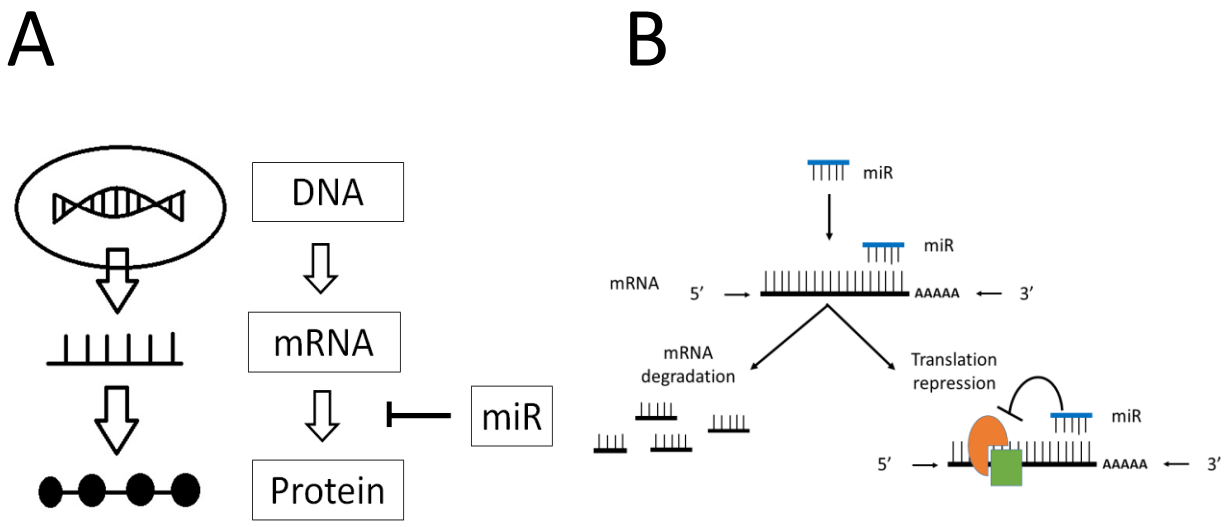

Figure 3-2 How miRs influence the process of protein synthesis.
miRs regulate which and how many of each mRNA are available to be translated into protein. Panel A shows where miR regulates mRNA in the protein synthesis process. DNA is transcribed into mRNA, which is then translated into protein. DNA consists of two strands of nucleotides linked together and wound around each other into a double helix shape found within the nucleus of the cell (the bowtie figure within the circle at the top). The four nucleotides of DNA are Adenine (A), Guanine (G), Cytosine (C), and Thymine (T). DNA is then reverse transcribed to create a single strand of nucleotides which will become mRNA (the single strand outside of the circle). The four nucleotides of RNA are Adenine (A), Guanine (G), Cytosine (C), and Uracil (U). mRNA is produced within the nucleus and then transported into the cytoplasm for translation. During translation, the mRNA sequence is used to determine which amino acids will make up the protein (the line with circles). Panel B shows how miR (blue single strand) regulates mRNA (longer black single strand), either through triggering mRNA degradation or repressing translation. When miR binds to mRNA it can trigger the intracellular mechanisms of target RNA-directed miRNA degradation. miRs can trigger translational repression (orange and green shapes on the black single strand) by binding to the target mRNA strand and blocking the translational machinery from continuing to translate the mRNA sequence.

miR in Zebra Finch Song Learning

miR may participate in establishing the patterns of gene expression necessary to support song learning in zebra finches (Kelly et al. 2018). Recent studies have found evidence for song experience-dependent fluctuations in large numbers of miRs in the auditory forebrain across age and sex (Dong et al. 2009, London et al. 2009; Gunaratne et al. 2011).

miR-2954 has been previously identified as being an interesting miR gene for investigation in zebra finches. miR-2954 was found to be expressed within the brain, and one of the mature miR produced from the gene, miR-2954-5p, demonstrated sex-specific changes in abundance after hearing song (Gunaratne et al. 2011; Lin et al. 2014). Within the auditory forebrain of adult zebra finches, hearing song playbacks resulted in no change in relative abundance of miR-2954-5p in males, but a significant decrease in the relative abundance in females compared to the expression levels of birds left in silence (Gunaratne et al. 2011)**.** While for the miR-2954-3p strand, hearing song playbacks resulted in an increase in the relative abundance in adult males and decreases in adult females compared to the expression levels of RNU6B, but neither of these changes were significant (Gunaratne et al. 2011)**.**

Knowing that miRs play a role in regulating mRNA available to be translated into protein combined with the requirement of new protein synthesis for learning and memory formation, I became interested in investigating whether the song responsive changes seen in adults emerge as a function of maturation and experience in each sex. I chose to investigate the role of these miR in each sex because these miR may be a molecular mechanism which regulates protein synthesis

within the auditory forebrain in juvenile male sensory song learning differently than those in juvenile female sensory song learning. To do this, I used a song playback paradigm to test how hearing song affects the relative abundance of these miRs - miR-2954-5p and miR2954-3p - within the NCM and CM sub-regions of the auditory forebrain of juvenile males and females. I wanted to test for changes in miR-2954-5p and miR-2954-3p across a known period of juvenile sensory song learning to be able to comment on how these results may impact the sensory song learning ability in each sex. I chose the juvenile male ability to memorize tutor song as the sensory song learning period to anchor my ages around because we know the ages of onset and offset are within the period of sensory song learning in juvenile males and females. Thus, I collected males and females at ages spanning the onset, P23 and P30, and the offset, P60 and P67. I used the *in situ* hybridization (ISH) technique to quantify the change in abundance of miR-2954-5p and miR-2954-3p after hearing song or being left in silence **(Figure 3-4).** This technique can be used to determine the neuroanatomical localization information of RNAs within brain tissue. I quantified the abundance of miR-2954-5p and miR-2954-3p within the NCM and CM because transcription and translation in juvenile males and females is not uniform across all sub-regions of the auditory forebrain (London and Clayton 2008; Ahmadiantehrani and London 2017; Bailey and Wade 2003).

For miR-2954-5p, I hypothesized that hearing song would result in no change in the relative abundance within the NCM and CM of juvenile males but would result in a decrease in the relative abundance within the NCM and CM of juvenile females. The reasoning behind this hypothesis is based on the song responsive changes seen in the auditory forebrain of adult males and females measured via real-time reverse transcription PCR (real-time RT-PCR) (Gunaratne et

al. 2011). Both adult males and females perform song recognition learning, and because of this I would expect hearing song playbacks to result in a significant decrease in the relative abundance of any miR which target the mRNAs of proteins necessary to support song learning. A decrease in the abundance of miRs implies there would be an increase in the diversity and abundance of the specific target mRNAs available to be translated into proteins. Thus, the significant song response changes in miR-2954-5p abundance in the auditory forebrain of adult females, but not in adult males, made me think that miR-2954-5p may be a female specific regulator of mRNA available to be translated into proteins that may contribute to the female ability to learn song (Gunaratne et al. 2011). Alternatively, miR-2954-5p may be regulating the translation of specific mRNA that are not involved in learning, rather mRNA that are necessary for female specific biological processes.

For miR-2954-3p, I hypothesized that hearing song would result in sex-specific changes in the relative abundance within the NCM and CM in juveniles when both sexes are capable of sensory song learning. The reasoning behind this hypothesis is based on the song responsive changes seen in the auditory forebrain of adult males and females measured via real-time PCR (Gunaratne et al. 2011). Studies have shown that both adult males and female exhibit similar molecular signatures of song recognition learning (Ahmadiantehrani et al. 2018). Juvenile males and females perform sensory song learning, but data suggest that juvenile males and females are doing molecularly distinct kinds of sensory song learning, corresponding to how both IEGs are expressed and how the phosphorylation of S6 is affected in response to song exposure (Ahmadiantehrani and London 2017; Bailey and Wade 2005; 2003). The song responsive changes in miR-2954-3p abundance were not significant in either adult males or adult females,

however, there does appear to be a visual difference between the two – miR-2954-3p abundance appears to be a 2X fold-change increase in Song compared to Silence in males but a 2X fold-change decrease in Song compared to Silence in females (Gunaratne et al. 2011). This visual difference in combination with the fact that molecular signatures of song learning are not the same across sex during development but are the same across sex in adulthood led me to think that perhaps miR-2954-3p is a mechanism with song-responsive sex differences during development that do not persist into adulthood. Thus, I hypothesize that hearing song will result in a significant increase in the abundance of miR-2954-3p within the NCM and CM of juvenile males and will result in a significant decrease in abundance within the NCM and CM of juvenile females.

If these predictions are correct, these patterns of miR-2954-5p and miR-2954-3p may indicate that these miR play a part in age- and sex-specific song responses that may contribute to juvenile sensory song learning. Mechanistically, I predict miR-2954-5p and miR-2954-3p are negative regulators of the transcription factor gene NR4A3. NR4A3 is one of the most strongly song responsive mRNAs in the zebra finch auditory forebrain and is a predicted target of miR-2954 (Dong et al. 2009; Warren et al. 2010; Lin et al. 2014). Inhibition of miR-2954 in a zebra finch cell line suppressed the expression of genes for proteins in the MAPK/ERK pathway (Lin et al. 2014). This is consistent with my mechanistic prediction that miR-2954 is a negative regulator of NR4A3, because a link between ERK signaling and the regulation of NR4A receptor expression has been shown across diverse tissue types and NR4A3 has been shown to be regulated by the MAPK pathway (Nomiyama et al. 2006; Stocco et al. 2002; Jennings et al. 2020).

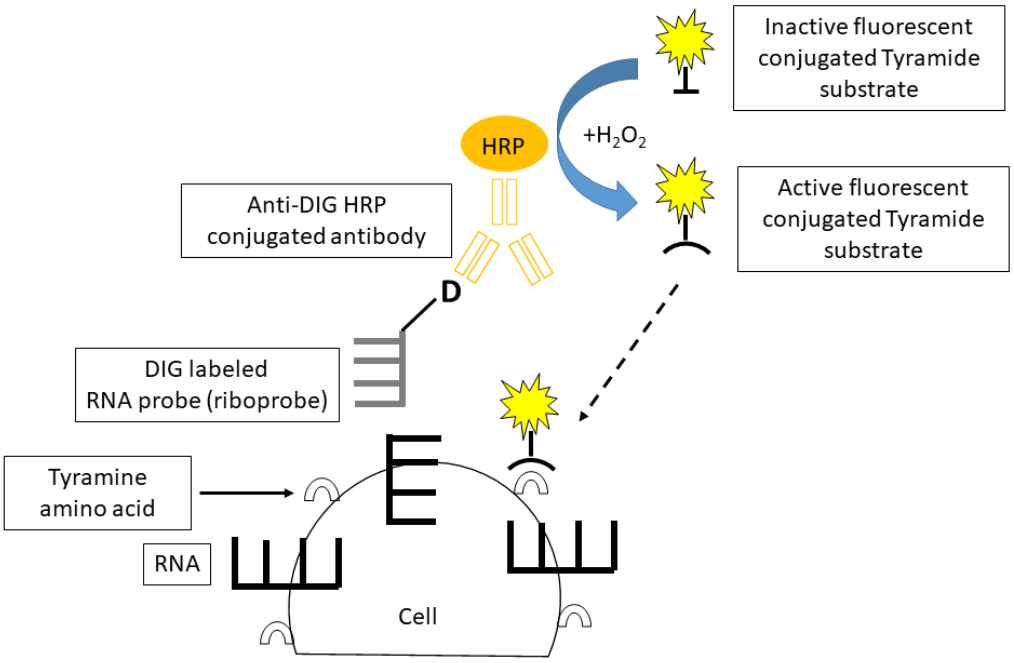

Figure 3-3 Diagram of ISH method.
ISH is a technique that uses specific RNA probes, called riboprobes, to determine the tissue distribution of a specific RNA. The riboprobe (gray strand) contains a reverse complement sequence of the target RNA (black strand), which allows the probe to bind specifically to the target RNA. The riboprobe is labeled with digoxigenin (DIG)-11-deoxyuridine triphosphates (shown here by the "D"). An anti-DIG antibody conjugated to horseradish peroxidase (HRP) (orange outline) can then bind to the DIG labeled riboprobe. The antibody-RNA complex can then be detected using tyramide signal amplification (TSA). With this detection method, the HRP enzyme (solid orange) binds to an inactive fluorescent conjugated Tyramide substrate (yellow star with black "T"). When hydrogen peroxide (H_2O_2) is present, HRP oxidizes the inactive Tyramide substrate into its active form (yellow star with black semicircle). Once in its active form, the fluorescent conjugated Tyramide can bind to the Tyramine amino acids (black outline semicircles) on the surface cells which contain the target RNA.

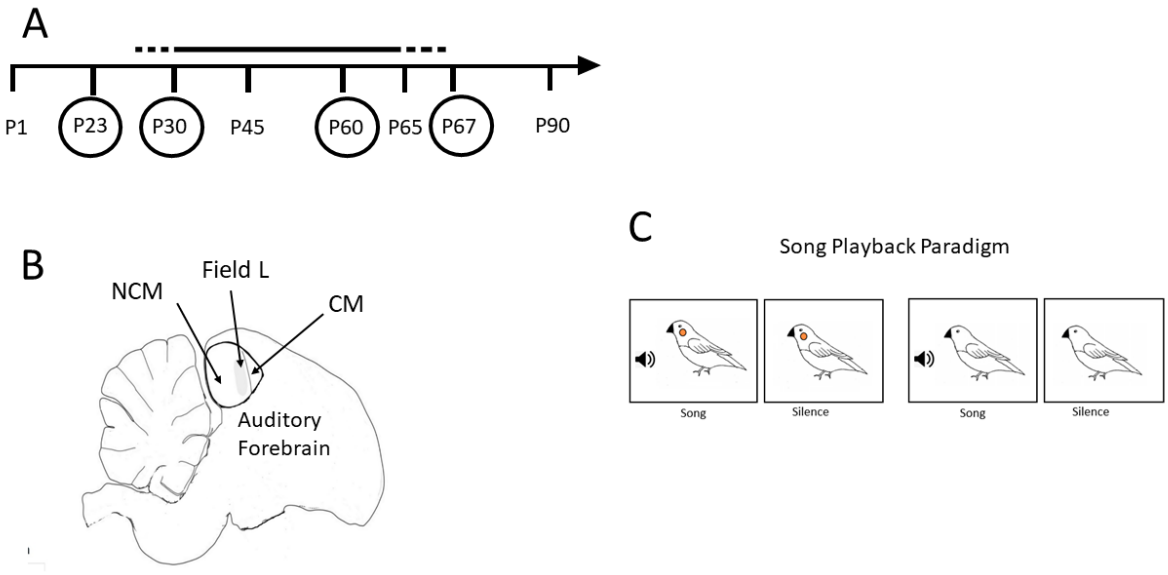

Figure 3-4 Experimental design.
Panel A depicts the ages of the "Normal" juvenile males and females which were collected. Six birds of each sex were collected at each age circled in black. Panel B depicts the ages of the "Tutored" and "Isolate" P67 males collected. Juvenile males (black beak with orange cheek patch) reared in the Tutored condition were reared with one adult male (orang beak with orange cheek patch) and one adult female (orange beak with no cheek patch). Males reared in the Isolate condition were reared with two adult females. Males were removed from the aviary at P23 and placed in temporary cages with other P23 juveniles and adult females until the males reached P30. At P30, the males were moved into their respective conditions. They remained in these conditions until P66 then were placed into soundproof chambers to be collected the morning of P67. The Tutored condition is outlined in blue and the dashed line begins at P23, the age at which the rearing condition for the Tutored birds becomes different from the Normal birds, includes a depiction of the different rearing environment, and concludes at P67 when these birds are collected. The Isolate condition is outlined in red to depict the same things. Panel C shows a depiction of the neuroanatomical location of the auditory forebrain and its sub-regions; NCM, CM, and Field L, within the zebra finch brain, adapted from Figure 3 in London 2019. Panel D shows a depiction of the soundproof chambers the birds were placed in overnight prior to the morning the bird was collected.

Methods

Subjects.

All procedures were conducted in accordance with the NIH guidelines for the care and use of animals for experimentation and were approved by the University of Chicago Institutional Animal Care and Use Committee (ACUP no. 72220).

Rearing.

All zebra finches were housed on a 14 hr:10hr light:dark cycle, with seed and water provided *ad libitum*. The juveniles used in this study hatched in flight aviaries, amongst males and females of all ages. The "Normal" juvenile males and females, 24 per sex, were raised in a breeding aviary and were tutored under normal social conditions (i.e., by their father or other adult males in the breeding colony). All birds remained in the aviary until the night prior to collection, at which point birds were moved into individual acoustic chambers 3 hours before lights off to facilitate the song playback paradigm the morning of collection.

Song Playback Paradigm.

Juvenile males and females were collected at P23, P30, P60 and P67. All experimental birds were either exposed to a song playback or left in silence. All birds were placed into a soundproof chamber overnight on the evening before they were to be collected. The following morning, 2 hours after lights came on, those in the song group heard 30 min of a song not heard previously ("Song" condition). Age-, sex -matched controls heard no song playback ("Silence" condition). There were three pairs of Song and Silence condition, n = 3, birds for all age- sex-combinations (Mello et al. 1992). Birds in the Song condition were exposed to novel conspecific

song, "zf101". This song was obtained from a bird bred and raised more than 20 years ago at the Ohio State University by Dr. Susan Volman and were thus novel to all subjects. The acoustic stimuli were played back through a speaker (model FE103, RadioShack) located in the chamber. The average sound intensity was adjusted to 70 dB via a sound pressure meter (model 33-2055, RadioShack).

At the end of the song playback or silence period each bird was sacrificed by rapid decapitation, and its whole brain was removed, placed in TissueTek O.C.T. Tissue Plus (Fisher HealthCare, cat. no. 4585), and rapidly frozen on dry ice. At the end of the song playback, all brains were transferred and stored at −80 ˚C until they were sectioned on a cryostat.

In Situ Hybridization (ISH).

The riboprobes used for biological and technical validation of ISH were produced via *in vitro* transcription of cloned DNA inserted in a plasmid. The plasmids containing the cloned DNA were from a collection of Expressed Sequence Tags (ESTs) from a normalized zebra finch brain cDNA library, verified via high throughput DNA sequencing (Replogle et al. 2008). One of the plasmids used in this experiment, pDC13, was not from the EST library, but rather was created by Dr. David Clayton's lab at the University of Illinois Urbana-Champagne. Once the correct plasmid was identified from the EST library, I grew up the bacteria which contained the plasmid in 6mL of Luria Broth (LB) with 100 µg/mL ampicillin overnight in a rotating incubator at 37˚C. The following morning, I created a purified plasmid solution by running a series of reactions in which the bacteria was lysed, the plasmid DNA was separated from the rest of the solution, and then purified through specific filtering spin column. All of these reactions were

carried out according to the instructions and using the reagents and spin columns within the Qiagen Miniprep Spin Kit (Cat# 27106). RNAse free *in vitro* transcription reaction (1µg/20µl DNA, 1X transcription buffer, 2mM DIG labeled NTPs, 2U/µl RNA polymerase converted the plasmid DNA into DIG-labeled riboprobes. The reaction was incubated for 2hrs in a 37˚C water bath. Once the reaction was complete it was placed on ice for three mins then immediately processed with the Qiagen RNeasy MinElute Cleanup Kit (Cat# 74204) to elute the DIG-labeled riboprobe from the rest of the cell lysate solution.

The riboprobes used for the ISH experiments investigating the abundance of miR-2954-5p and miR-2954-3p were a specific type of riboprobe which were created using locked nucleic acid (LNA) and do not require *in vitro* transcription of cloned DNA. All unlabeled LNA oligonucleotides (Exiqon) were digoxigenin (DIG) labeled by the DIG Oligonucleotide Tailing Kit, 2nd Generation (Roche, cat. no. 0335383910). This kit adds DIGs to the –OH on the 3' end of the oligonucleotides.. The labeling efficiency was estimated using a dot blot. The protocol for using LNA probes and Tyramide Signal Amplification (TSA, Life Technologies, cat. no. T20932) to detect microRNA in frozen tissue sections was followed according to a previous study, which was optimized accordingly (Lin et al. 2014). miR-124 was used as a positive control. miR-124 is an abundant, brain-specific miR and it been implicated in the regulation of synaptic plasticity and memory formation (Malmevik et al. 2016; Michely et al. 2015; Wang et al. 2022). A miR probe with a scrambled miR-2954 sequence was used as a negative control.

These LNAs were the same, in length and in sequence, as the miR-2954-5p and the miR-2954-3p probes.

The negative control probe was designed to not align to any sequence within the most recent zebra finch genome, NCBI genome annotation corresponding to the bTaeGut1.4.pri assembly (GCA_003957565.4) (Rhie et al. 2021). The sequence of the negative control probe was selected from Qiagen's GeneGlobe miRCURY LNA miRNA Detection Probes (https://geneglobe.qiagen.com/us/product-groups/mircury-lna-mirna-detection-probes). The negative control sequence was checked with BLAT (BLAST-like alignment tool), which aligns the negative control sequence to the zebra finch genome, NCBI genome annotation corresponding to the bTaeGut1.4.pri assembly (GCA_003957565.4), using University of Santa Cruz's Genomic Institute Genome Browser (UCSC) (Kent 2002, Rhie et al. 2021). The LNA probe technology produces probes that can discriminate between two RNA sequences with overlap as low as two nts. This means that when the negative control probe was aligned to the zebra finch genome, as long as there were three nts or more of the probe that did not align with the sequences in the genome, it would be an efficient negative control.

Parasagittal brain sections were collected, 12µm in thickness, on a Thermo NX50 Cryostat across the entire brain. Sections were thaw-mounted onto SuperFrost Plus glass slides (Fisher Scientific, cat. no. 12-550-15) and stored at −80 ˚C until use. Sections were removed from −80 ˚C storage, fixed with 4% (wt/vol) Paraformaldehyde (pH 7.4) in double distilled water for 15 min, washed three times in 0.025M KPBS (0.02M Dibasic Potassium Phosphate, 0.003M Monobasic Potassium Phosphate (KH_2PO_4), 0.15M Sodium Chloride) for five mins each, treated

with 0.1M Triethanolamine for three mins, treated with 0.25% (vol/vol) Acetic Anhydride/0.1 M Triethanolamine for 10 min, washed twice with 2X SSC (0.15M Sodium Chloride and 0.017M Sodium Citrate), and dehydrated with a series of three min Ethanol washes (75%, 90%, and 100% Ethanol). All pre-hybridization steps were done in RNase free conditions. Slides were allowed to dry for one hour prior to hybridization with 2.5 pmol DIG-labeled LNA probe in hybridization buffer [50% (vol/vol) Formamide, 500µg/ml yeast tRNA, 300 mM NaCl, 20 mM Tris (pH 8.0), 5 mM EDTA (pH 8.0), 1X Denhardt's, 10 mM NaH_2PO_4 (pH 8.0)] was carried out for 16 h at 65 ˚C followed by a wash in 50% (vol/vol) Formamide, 1X SSC (15mM NaCl, 1mM Tris (pH 8.0) and 0.25mM EDTA (pH 8.0)) and a serial washes with 0.1X SSC (1.5mM NaCl, 0.1mM Tris (pH 8.0) and 0.025mM EDTA (pH 8.0)) at 65 ˚C.

Chromogenic visualization

Sections were then washed with GBA (1M Tris pH 7.5, 5M NaCl) three times for three mins each. Sections were then incubated in 4µl anti-DIG-AP FAB fragments antibody (cat# 11093274910) in 20ml of blocking solution for 2.5hrs at room temperature. Sections were then washed four times in GBA for five mins each then washed in GBB (1M Tris pH 9.5, 5M NaCl, 1M $MgCl_2$) for 10 mins. Sections were then covered with 500µl of NBT/BCIP solution (1 pellet of NBT/BCIP dissolved in 10ml of ddH_20) until purple color began to develop. Color reaction was stopped with ddH_20.

TSA visualization

Sections were then treated with 3% Hydrogen Peroxide in TSA Amplification Buffer (Invitrogen, cat. no. T20932) for 30 min, washed with TN buffer (0.1 M Tris-HCl,

pH 7.5 and 0.15 M NaCl) for two mins and then incubated in Blocking Buffer [0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.5% (wt/vol) blocking reagent (Roche, cat. no. 11096176001) and 0.5% (wt/vol) BSA] for 30 min at room temperature, and then washed three times for five mins each in TN buffer. Sections were then covered with 100 µl of tyramide signal amplification (TSA) and placed in a light-proof chamber for 30 mins. After 30 mins, sections were removed from the light-proof chamber, washed three times for five mins each in TN buffer, cover slipped with PVA mounting medium (1M Tris Base, pH 9, MilliQ water, PVA (Sigma Type II polyvinyl alcohol P-8136), Glycerol, 1% Merthiolat), and kept in a light-proof slide box at 4˚C until imaged.

Immunohistochemistry

Sections were permeabilized with 0.1 M PBS containing 0.03% Triton-X on a rotator at room temperature for 30mins and then washed with 0.1 M PBS containing 0.5% Tween-20 (PBST) for 10 mins at room temperature three times. Sections were blocked for 1hr at room temperature in 2% normal goat serum (NGS; Cat# NC9270494, Vector Laboratories, Burlingame, CA, USA). A monoclonal mouse IgG anti-NeuN primary antibody (1:500 in 1% NGS; #MAB377, EMD Millipore, Billerica, MA) was applied to the sections for overnight incubation (~16hr) at 4°C. Sections were then washed with PBST for 10mins at room temperature three times and incubated in Biotinylated Goat anti-mouse IgG secondary (1:200 in 1% NGS; Cat# A-16070, Thermo Fisher Scientific, Waltham, MA) for 1hr at room temperature. Sections were washed with PBST for 10 mins at room temperature three times. After washing with PBST, sections were incubated in avidin-biotin complex (Vectastain Elite ABC Kit; Vector Laboratories) for 30 mins at room temperature. The peroxidase complex was visualized with

3,3′-Diaminobenzidine (DAB, Sigma, St. Louis, MO, USA) containing 0.003% H_2O_2 in 0.1M PBS. Sections were then mounted, dehydrated, cleared, and coverslipped with Permount (Fisher Scientific).

Image Acquisition and Processing.

All images were captured using a Zeiss Axiovert 200M microscope with a Zeiss Axiocam digital color CCD camera (Carl Zeiss Microscopy, Thornwood, NY, USA) running Slidebook 5.5 software (Intelligent Imaging Innovations, Denver, CO, USA). Each Superfrost Plus glass microscope slide held five serially cut sections. Slides with tissue including the auditory forebrain, spanning from midline to ~990µm lateral, were used for most of the experiments. Slides with lateral tissue, ~990µm later to ~2.5mm, were used for the later experiments to try and salvage this project. Tissues from both juveniles and adults were used. For all brain sections, we obtained images that contained all three major sub-regions of the auditory forebrain; Field L, NCM, and CM, plus the adjacent hippocampus (HP) with a 5X objective. Neuroanatomical landmarks were identified using the Histological Atlas on the Zebra Finch Expression Brain Atlas (ZEBrA, Oregon Health and Science University, Portland, OR, USA: zebrafinchatlas.org) as a reference. These landmarks were visible using the 5X objective and used to quantify NCM, CM, and HP across brain sections and individuals. The landmarks for the NCM are as follows; dorsal boundary landmarks = hippocampus, area parahippocampalis (APH), and lateral ventricle, posterior boundary landmarks = cerebellum and posterior telencephalon, ventral boundary landmarks = choroid plexus (ChP) and septum, anterior boundary landmarks = Field L. The landmarks for the CM are as follows; dorsal and anterior boundary landmarks = area parahippocampalis (APH), posterior boundary landmarks = Field L,

ventral boundary landmarks = ChP, nidopallium. The landmarks for Field L are as follows; dorsal, posterior, and ventral boundary landmarks = L2a (one of the cytoarchitectonic subdivisions of the neostriatal field L complex in caudal telencephalon), anterior boundary landmarks = lamina hyperstriatica (LH)

pS6+/S6+ cell density was quantified within the NCM and CM and was normalized to the values from the adjacent hippocampus (HP), using ImageJ (Mello and Clayton 1994). Each image was opened in ImageJ and the region of interest was manually circled with the freehand selection tool. The image was then transformed into an 8-bit image and the threshold was adjusted to most accurately highlight the cells within the region of interest. The area, standard deviation, integrated density, limit to threshold, mean gray value, and modal gray value were all measured and recorded. Using the invert table (a table where you can invert the color of the image without changing the pixel value) the image was then flipped via "Invert LUT". This was done in order for the ImageJ algorithm to easily distinguish cells from background, and the number of cells were recorded in pixels^2 with the minimum size requirement of three pixels^2. All measures were corrected for the size of the measured area. The number of labeled cells within the HP was quantified to confirm that the staining within does not change systematically with sex, age, or song exposure before using it as technical control. The pitfall of imaging this way is that it introduces human error via the manually drawn region of interest. Thus, all NCM and CM measurements were corrected for area and normalized to the HP measurements. Also, a random

number generator was used to determine the order of images to be analyzed in order to minimize the potential for systematic human error.

Statistics.

We would have analyzed the normalized NCM and CM values from the ISH with three-way ANOVAs (SPSS; $\alpha = 0.05$) to test for main effects of age, acute song experience, rearing condition, and sex, and their corresponding interactions.

Results and Discussion

Establishing Positive Controls for ISH

The experimental design included utilizing the ISH technique to detect differences in the abundance of miR-2954-5p and miR-2954-3p across age, sex, and acute experience. Prior to running the experiment, positive biological and technical controls needed to be established which would provide the evidence to support the use of the ISH protocol to answer the hypotheses.

For the initial ISH, the visualization method used alkaline phosphatase-labelled anti-DIG-antibody and nitroblue tetrazoliumchloride and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP), more commonly known as chromogenic ISH or CISH. Digoxigenin (DIG)-labelled RNA probes (riboprobes) were produced in order to perform CISH. The tissues used during these initial ISH experiments were from adult males and females. Adults were used because of availability of information about the song responsive changes in gene expression within the

auditory forebrain of both sexes (Gunaratne et al. 2011, Wada et al. 2004, Bolhuis et al. 2000; Mello et al. 1995; Mello and Clayton 1994)

Technical Control

The goal of producing a technical control was to show that ISH could be used to detect the neuroanatomical localization of any probe used. The probe I selected for my technical control was for NR1. NR1 is an N-methyl-D-aspartate receptor (‘or NMDAR’) subunit. NMDAR is a glutamate receptor and ion channel ubiquitously expressed on neurons in the auditory, anterior forebrain, and motor pathways within zebra finches (Wada et al. 2004). NR1 is a ubiquitous receptor subunit in the brain, and two of its eight splice variants, NRla and NRlb, are the most abundant mRNAs coding for NMDA receptors in mammalian brain cells (Kope et al. 1993). NR1 is the requisite subunit for all NMDAR, you cannot have a functional NMDAR without an NR1 protein (Atlason et al. 2007). This means that an ISH using the NR1 probe should produce selective binding of the probe across the brain – regardless of age, sex, or acute song exposure. An ISH result of a lack of observable signal could mean that the mRNA target is not expressed; but with the use of a probe for a ubiquitous receptor subunit, like NR1, a lack of observable signal would indicate a technical issue with the protocol despite the presence of the target. Another strength of the NR1 clone was its size: 520 nts long. Previous studies have found that riboprobes around 500 nts are optimal to use for ISH because their length allows them to have more DIG molecules to enhance sensitivity, while not being so long that the length inhibits the riboprobes ability to penetrate the tissue (Bromley et al. 1994; Brown et al. 1994; Coker et al., 1996). I was able to generate a NR1 riboprobe from clone DV954900, an existing clone within

the EST library and successfully produced the expected results – expression across the auditory forebrain (Replogle et al. 2008) (**Table 3-1**).

Biological Controls

The goal of producing a biological control was to show that ISH could be used to detect a difference in the neuroanatomical spatial localization of a gene in birds that had received an acute song experience or had been left in silence. The gene I selected was *egr-1,* one of the genes included in *ZENK (zif268, egr-1, ngfi-a, krox24).* I selected *egr-1* because of the difference in expression between adult males that had received an acute song experience or had been left in silence – it is ubiquitously and robustly expressed within the NCM and CM of adult males after hearing song playbacks compared to the little to no expression in adult males left in silence (Bolhuis et al. 2000; Mello et al. 1995; Mello and Clayton 1994). Prior to the COVID-19 lockdown, I successfully generated an *egr-1* riboprobe from a pDC13 clone previously made by Dr. Sarah London. Hybridization with it produced the expected results – significantly more *egr-1* expression within the auditory forebrain of Song tissue compared to the auditory forebrain of Silence tissue (p-value = 0.01) **(Figure 3-5)**.

Upon returning to lab following the COVID-19 lockdown, I could not reproduce the *egr-1* results. There is no biological explanation for this; I used the same tissue and reagents. During the lockdown, the lab was moved to an entirely new space multiple floors above where it used to be. However, this should not have had any influence on these results because it was still the same equipment being used. We didn't feel comfortable moving forward with the experimental plan

until we could successfully produce a biological control, so we decided to generate a riboprobe for NR4A3. NR4A3 (or nuclear receptor subfamily 4, group A, member 3 - (also known as neuron-derived orphan receptor-1 (NOR-1)), is a nuclear receptor that has been found to have high levels of mRNA in the CNS (Kurakula et al. 2014; Bookout et al. 2006). NR4A3 is one of the most strongly song responsive mRNAs in the auditory forebrain of adult males (Dong et al. 2009; Warren et al. 2010). I generated a NR4A3 riboprobe from the existing clone found in the EST library, FE725799, which successfully produced the expected results after hybridization – significantly more NR4A3 expression within the auditory forebrain of Song tissue compared to the auditory forebrain of Silence tissue (p-value = 0.02) (Replogle et al. 2008) **(Figure 3-5)**.

Establishing Positive Controls for Fluorescence ISH

The visualization method of the ISH experiments had to change because the miR probes I would be using in the experiment are too short to be visualized with NBT/BCIP. Fluorescence detection is more sensitive for shorter probes than using NBT/BCIP as the detection mechanisms.. This changed the protocol slightly to accommodate a fluorescence method of visualization, more commonly known as FISH. The main difference between CISH and FISH is that instead of utilizing an alkaline phosphatase-labelled anti-DIG-antibody to catalyze the NBT/BCIP reaction, FISH uses a fluorescence-labelled anti-DIG-antibody which allows for a fluorescent signal to be given off. The amount of time necessary for an NBT/BCIP reaction varies significantly and can take hours to fully develop. FISH tissue needs only minutes to amplify the fluorescent signal to a degree that can be detected The majority of the ISH protocol is the same between these two visualization methods; however, it was important to demonstrate

that positive controls could be reproduced utilizing FISH. I used the NR4A3 riboprobe I previously generated using the FISH protocol and successfully produced the expected results – significantly more NR4A3 expression within the auditory forebrain of Song tissue compared to the auditory forebrain of Silence tissue using FISH (p-value = 0.01) **(Figure 3-5)**.

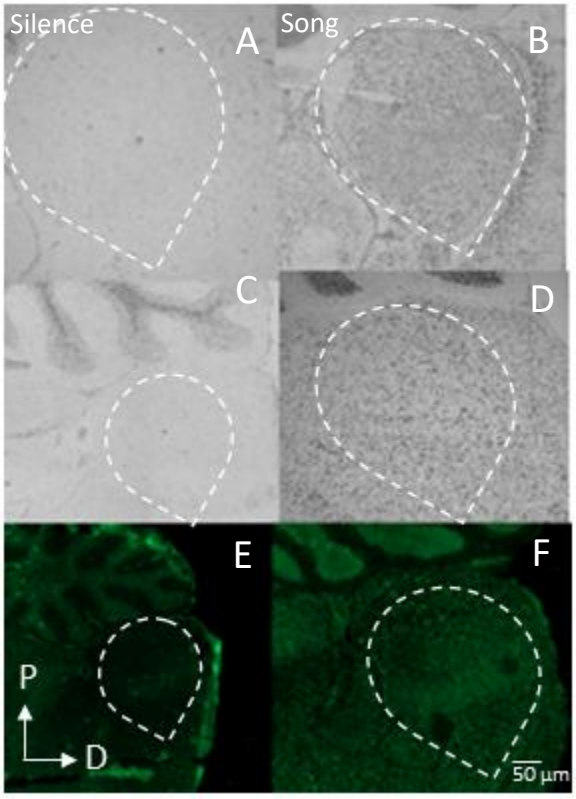

Figure 3-5 Establishing positive biological controls for ISH using both the BCIP/NBT and fluorescent methods of visualization.
This figure shows the results of ISH experiments with the two positive biological control probes on adult male tissue. A successful biological positive control would result in little to no signal within the Silence tissue and a significant increase in signal within the Song tissue when compared to the Silence tissue. The left-hand column, comprised of panels A, C, and E, shows images of auditory forebrain tissue from an adult Silence male. The right-hand column, comprised of panels B, D, and F, shows images of auditory forebrain tissues from an adult Song male. Panels A and B are from an ISH using a *ZENK* probe with the BCIP/NBT visualization method. Panels C and D are from an ISH using a NR4A3 probe with the BCIP/NBT visualization method. Panels E and F are from an ISH using NR4A3 with the fluorescent visualization method. The auditory forebrain is outlined in white. An orientation axis is included in the lower left-hand corner: P = posterior and D = dorsal. A scale bar is included in the lower right-hand corner denoting 50µm. All images were taken with the 5X magnification lens.

FISH with Selected miR Probes

For the main ISH experiments, I used miR probes. The miR probes are different from the riboprobes in two ways. First, they are drastically different in size. The riboprobes are roughly 500 - 2600 nts long, whereas the miR probes are around 20 nts long. The difference in probe size is significant; longer probes exhibit higher specificity than shorter probes. The longer the probe sequence, the greater the number of nucleotides available to bind to the target sequence. Using a shorter probe equates to a smaller margin of error when performing the ISH experiment, because there are fewer nucleotides available to bind to the target sequence. An important caveat, while the LNA probes are shorter, they are not directly equivalent to a non-LNA riboprobe of the same length. This is because of the added binding kinetics that are intrinsic to LNA probe design, which I discuss below. Second, these two types of probes are generated in different ways and are constructed differently. In the experiments up to this point, riboprobes were generated via *in vitro* transcription. The miR probes were purchased and then DIG labeled in the lab. These probes were locked-nucleic acid probes, or LNAs. LNA probes use modified RNA nucleotides in which the ribose moiety is modified with an extra bridge connecting the 2' oxygen and the 4' carbon (Darnell and Antin 2016). This modification allows for thermal stability when hybridized to RNA, for each LNA monomer incorporated the melting temperature (Tm) of the probe-sequence duplex increased by 2-8˚C (Jensen et al. 2011). The LNA probes maintain this characteristic regardless of probe size, which is an important feature when detecting small targets, such as miR. The use of LNA probes have been effective in previous FISH experiments within the auditory forebrain of adult male and female zebra finches (Lin et al. 2014).

Four different miR LNA probes were used. The two probes that were foundational in developing this project, miR-2954-3p and miR-2954-5p, miR-124 as a positive control, and a scrambled oligonucleotide sequence as the negative control (Rajasethupathy et al. 2009; Yang et al. 2012; Hu and Li 2017; Wang et al. 2012; Wang et al. 2021; Aksoy-Aksel et al. 2014; Thomas et al. 2018). The miR-2954-3p and miR-2954-5p probes included the same sequence and purchased from the same supplier that was used in Lin et al. 2014. miR-124 was selected as the positive control because miR-124 is an abundant, brain-specific miR that has been found to have a ubiquitous expression pattern in most neuronal cell types and to have a prominent role in the negative regulation of synaptic plasticity and memory formation (Sun et al. 2015; Rajasethupathy et al. 2009; Wang et al. 2022; Yang et al. 2012). The negative control probe (scrambled miR) was purchased with the same length and composed of the same number of LNA nucleotides as the other miR probes being used. The purpose of a negative control was to be able to measure the level of background or noise produced from the FISH visualization. The expectation is that this probe will produce little to no signal when it is used.

Optimizing the FISH protocol

Following the protocol published in Lin et al. 2014, an ISH experiment was run with all four of the selected miR probes on adult male brain tissue cut at a width of 12µm in the sagittal plane from midline to ~990um from midline to ensure the auditory forebrain was included in the tissue sections. This experiment resulted in signal from all four probes **(Figure 3-6)**. This was a problem because there should not be signal from the negative control.

Initially I thought that the negative control purchased may no longer be appropriate given that it had not been aligned with the most current zebra finch genome, NCBI genome annotation corresponding to the bTaeGut1.4.pri assembly (GCA_003957565.4) (Rhie et al. 2021). When I BLAT the negative control sequence against the genome assembly GCA_003957565.4, the search indicated that there was 11/22 nts overlap between the negative control sequence and the genome assembly. Even though half of the negative control sequence overlapped with sequences within the zebra finch genome, the probe will not bind to any zebra finch tissue because the probe has 11 nts that do not align and it only needs 2 nts to not align to prevent binding. With this information, I reached out to Qiagen's GeneGlobe miRNA LNA development team to get their opinion on what could be the problem with my ISH. They suggested that the signal I was seeing from my negative control was likely due to utilizing an inappropriate hybridization temperature **(Figure 3-7.2)**. The protocol I was following called for a hybridization temperature of 60˚C. The DNA-Tm (or the DNA annealing temperature) of these miR probes is 77.6˚C. Thus, I interpreted the signal I was seeing from all four of my probes was non-specific: at 60˚C my probe was binding to both DNA and RNA. RNA can bind to DNA because, while they have different sugars in the backbone of the chain, three of the four nucleotides are identical. The fourth nucleotide is thymine in DNA and uracil in RNA, which have very similar chemical structures. A hybridization temperature above 76˚C is necessary for specific RNA-RNA binding because at this high temperature three out of four nucleotides binding between the probe and the tissue is not enough to stabilize the bonds between the probe and the tissue, i.e. this is a high enough temperature to require binding across all nucleotides in order for the probe to anneal. The RNA-Tm, or the RNA annealing temperature, for my three positive control miR probes is 83.7

˚C and the RNA-Tm for the negative control is 87.3 ˚C. The ideal hybridization temperature to produce specific miR signal would be between 78 ˚C – 83 ˚C.

I ran an ISH experiment with all four of miR probes with a hybridization temperature of 80 ˚C. This resulted in no signal from any of my four probes (**Figure S1**). I reached back out to the manufacturer, and they sent me the protocol they recommended using with LNA miR probes. The major difference between the protocol I was following and the protocol I was given was the inclusion of a Proteinase K solution incubation immediately prior to applying the hybridization solution **(Figure 3-7.3)**. Their protocol stated that due to the size of miRs Proteinase K may be needed to demask, or catalyze the proteolysis of, membrane proteins, translational machinery, and ribosomal proteins. The proteolysis of membrane proteins is important for the probe to be able traverse the cell and/or the nucleus membrane to find the target miR within the cell. The proteolysis of translational machinery and ribosomal proteins is important for the probe to be able to bind to the miR. Optimized Proteinase K solution treatment is essential to enable the maximum amount of probe to be able to bind to the miR target; accordingly, I completed a series of pilots to determine the appropriate concentration and duration (**Table 3-1**). The criteria for determining what combination of concentration of Proteinase K solution and duration on the tissue was decided based off how damaged the tissue looked after being treated with the Proteinse K solution. The 'miRCURY LNA miRNA Detection Probe Handbook' provided a range of concentrations and durations to test. I chose to test 8 of these combinations. Any concentration of Proteinsase K greater than 0.015mg/ml or a duration longer than 10 mins resulted in visible tears in my tissue. Thus, I determined that the appropriate combination of

concentration and duration was 0.015mg/ml for 10 mins. Once determined, there was still no signal with any of the four probes (**Figure S2**).

Based on the ISH protocol, there was no other biological explanation for why I was not able to produce the expected results that I had not yet explored, so I evaluated the hybridization oven that I was using **(Figure 3-7.4)**. This particular piece of equipment was roughly 15 years old, and I had not measured if it was capable of maintaining a specific temperature overnight. As it turns out, the oven was heating up and cooling down drastically overnight. With the oven set at 80˚C it was heating up above 88˚C at times and cooling down below 78˚C at times. This is a problem, because the RNA annealing temperature for my probes is approximately 78˚C and the RNA melting temperature for my probes is approximately 88˚C. The oven heating up above 88˚C prevents optimal binding of the probe to the tissue, while the oven cooling below 78˚C leads to non-specific binding – meaning my miR probes could be binding DNA or RNA. I then tried to find a "sweet spot" on the oven's temperature dial in which the majority of the time the internal temperature would stay between the RNA annealing and melting temperatures, but was unable to do so. Before thinking about purchasing a new hybridization oven, I wanted to verify that a hybridization temperature of 80˚C would in fact produce the results I was looking for – signal from the positive control and no signal from the negative control. In order to do this, I reached out to other labs to see if there was a hybridization oven I could use that was able to maintain 80˚C overnight. I was able to find an oven that met these parameters which produced the expected results (**Figure S3**). After replicating these results, it was clear that we would need to purchase a new oven in order to run my main experiment.

Once the new oven had arrived, I ran an ISH with a hybridization temperature of 80˚C which resulted in no signal from either my positive or negative controls (p-value = 0.67) (**Figure S4**). I checked to make sure the oven was in fact maintaining temperature overnight, and it was, but the temperature depended on where the rack was placed within the oven (**Table 3-2**). Once I had determined where the rack must be located to hold 80˚C overnight, I was able to run an ISH which produced the expected results – signal within the auditory forebrain from the miR124, but not from the negative control (p-value = 0.03) (**Figure S5**).

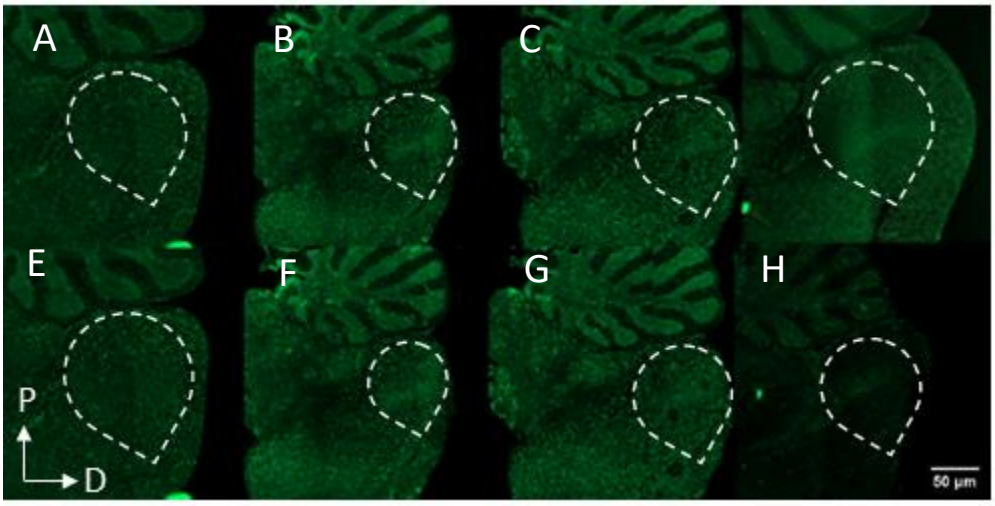

Figure 3-6 Comparison of ISH results following the protocol from Lin et al. 2014 to the expected ISH results.

The ISH experiment consisted of all four miR probes with a hybridization temperature of 65˚C on adult male tissue. There was signal produced from all four probes. Panels A-D show the initial results from the ISH I ran following the protocol published in Lin et al. 2014. Panels E-H show the expected ISH results – signal within the auditory forebrain from the miR-2954-3p, miR-2954-5p, and miR-124 probes and no signal from the scrambled probe. The images of the expected ISH results were produced after I had adjusted and optimized the protocol from Lin et al. 2014. Optimizing the protocol included the addition of a Proteinase K step and adjusting the hybridization temperature. Panel A and E show images of the miR-2954-5p probe. Panel B and F show images of the miR-2954-3p probe. Panel C and G show images of the miR-124 probe. Panel D and H show images of ISH using the scrambled miR probe. The auditory forebrain is outlined in white. An orientation axis is included in the lower left-hand corner; P = posterior and D = dorsal. A scale bar is included in the lower right-hand corner denoting 50µm. All images were taken with the 5X magnification lens.

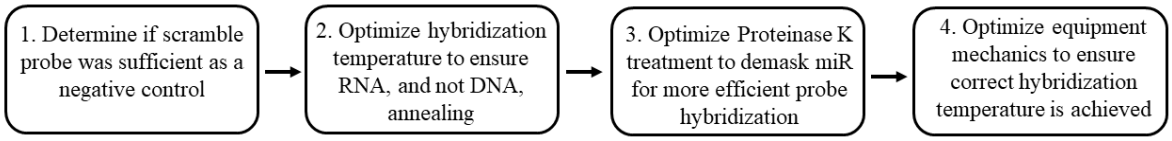

Figure 3-7 Steps taken to optimize the FISH protocol.
These are the steps that were taken to optimize the ISH protocol after the initial experiment produced signal from all four of the probes – including the negative control. After completing these four steps, the expected results were produced – signal from all probes except the negative control.

Table 3-1 Proteinase K optimization parameters.

Concentration of Proteinase K	Duration on the tissue	Concentration of probe
0.015 mg/ml	10 mins	5 pmol/µl
0.015 mg/ml	15 mins	5 pmol/µl
0.015 mg/ml	20 mins	5 pmol/µl
0.015 mg/ml	10 mins	10 pmol/µl
0.015 mg/ml	15 mins	10 pmol/µl
0.015 mg/ml	20 mins	10 pmol/µl
0.03 mg/ml	5 mins	10 pmol/µl
0.03 mg/ml	10 mins	10 pmol/µl

This table includes the various Proteinase K solutions I used on the tissue to try and increase the binding efficiency of the experimental probes to the tissue. I varied the concentration of Proteinase K, the duration that the solution was on the tissue, and the concentration of probe applied to the tissue after the Proteinase K solution treatment.

Table 3-2. Hybridization oven rack temperature optimization parameters.

Rack Number	Temperature on rack in the evening	Temperature on rack in the morning
1	74.5˚C	74˚C
2	79.5˚C	80˚C
3	83.5˚C	83˚C

This table includes the temperature readings across all of the oven racks within the hybridization oven when the built-in oven thermometer is set to 80˚C.

This experiment only included one hybridization tray with two slides within it, one positive control and one negative control. For the actual experiment, in order to keep the groups balanced within each tray, I would need to run an ISH with 12 slides per tray, eight hybridization trays total. First, I re-ran the previous experiment with the addition of 10 dummy sides, SuperFrost Plus slides with no tissue on them, within the tray to see if the added slides would affect the hybridization ability of my probes. This experiment produced the expected results – signal from my positive control but no signal from the negative control. Next, I duplicated the previous experiment but included 8 hybridization trays all set up in the same way. Only one tray from this experiment produced the expected outcome; the other trays either had signal from both probes, or signal from neither probe (Fig S6 p-value = 0.72, Fig S7 p-value = 0.02, Fig S8 p-value = 0.66) (**Figure S6-8**). I ran a number of pilots to determine the appropriate rack height and number of hybridization trays per rack to ensure that each tray was maintaining 80˚C overnight. Once I determined the appropriate configuration, I was able to run an ISH that produced the expected result within each hybridization tray (**Figure S9-11**; tray 1 p-value = 0.02, tray 4 p-value = 0.01, tray 8p-value = 0.04). After duplicating this result, I decided to move on to the main experiment.

Running the Experiment

The main experiment would consist of running an N = 3 for each miR probe and tissue type combination spanning one hemisphere of the auditory forebrain. In total, the entire experiment would require 1,200 slides of tissue to be run. In order to make this more manageable and to create a balanced experimental design, I decided I would run N = 1 with one of my miR

probes, or 11 hybridization trays with 12 slides each, at a time. I decided I would start with the positive control, miR-124. The result of running the N = 1 with the miR-124 probe was no signal on any of the tissues. In order to conserve experimental tissue but also check the efficacy of the rest of my miR probes, I ran lateral tissue corresponding to the N=1 with miR-2954-5p and the miR-2954-3p miR probes. This also did not produce signal on any of the tissue. I ran an ISH with lateral tissue to the N = 2 and 3 with all of the miR probes and this also produced no signal from any of the probes.

I ran a side-by-side ISH with lateral tissue from N = 1 and lateral tissue from the practice tissue I had been using with the positive and negative control **(Figure 3-8.1).** This resulted in no signal on any of the experimental tissue, but the expected results on the practice tissue. I re-ran this experiment with lateral tissue from N = 2 and 3 compared to the practice tissue and this produced the same result - no signal on any of the experimental tissue, but the expected results on the practice tissue. I decided I would run an ISH with lateral tissue from N = 1, 2, and 3 compared to the practice tissue with the positive and negative controls at a hybridization temperature below the DNA-Tm **(Figure 3-8.2)**. I did this to further characterize what could be going wrong with the subsequent experiments. This final experiment produced no signal from either of the probes on the experimental tissue and showed signal from both probes on the practice tissue, as expected. At this point, I came to the conclusion that I would no longer be able to use the tissue that had been collected and processed for this experiment with this particular FISH technique.

Attempting to Salvage Experimental Tissue

In an attempt to find a use for the tissue that had been collected and processed, I ran a series of ISH with larger riboprobes I had generated from existing clones as well as immunohistochemistry (IHC) experiments. I thought that these experiments would allow me to target larger molecules utilizing more robust techniques. First, I ran an ISH with lateral tissue, tissue that does not include auditory forebrain, from N = 1, 2, and 3, and compared it to the practice tissue using the NR4A3 riboprobe **(Figure 3-8.3)**. This resulted in no signal on any of the experimental tissue and the appropriate signal on the practice tissue (**Figure 3-9**). Next, I ran an ISH with lateral tissue from N = 1, 2, and 3, and compared it to the practice tissue using the NR1 riboprobe. This resulted in no signal on any of the experimental tissue and the appropriate signal on the practice tissue (**Figure 3-9**). I re-ran the previous experiment, ISH with lateral tissue from N = 1, 2, and 3, and compared it to the practice tissue using the NR1 riboprobe, with the adjusted concentration and duration of my fixation step, and none of these produced signal on the experimental tissue (**Table 3-3**). Finally, I ran an immunohistochemistry (IHC) lateral tissue from N = 1, 2, and 3, and compared it to the practice tissue using NeuN (Fox-3, Rbfox3, or Hexaribonucleotide Binding Protein-3) **(Figure 3-8.4)**. NeuN is a neuronal specific nuclear binding protein in vertebrates and is the most commonly used protein biomarker for mature neurons (Mullen, Buck, and Smith 1992). This resulted in no signal on any of the experimental tissue and the appropriate signal on the practice tissue (**Figure 3-9**). When these experiments did not produce the expected results in the experimental tissue, it suggested to me that I may not be able to use any of this tissue in any meaningful molecular experiments given my current knowledge and the time in which I had left to complete all of my experiments.

Conclusions

In conclusion, I learned that long-term storage of 12µm sections on slides in slide boxes in a -80˚C freezer, a common and widely used strategy for long-term storage of tissue, may not be the best preservation method for tissue destined for ISH or IHC. This does not mean that this method of preserving the tissue is invalid, rather it means that I was not able to validate or invalidate my assumption that this preservation method may have contributed to my lack of results. It was never my intention for the tissue to be stored for such a long time, but the COVID lock-down caused me to step away from this experiment for a long time. If I were to do this project again, I would section the tissue for each N prior to running the experiment to minimize the amount of time the tissue would spend in the -80˚C.

Reflection on Lin et al. 2014

Given the time I have spent troubleshooting this FISH experiment and the experts I have consulted, the images and results published in Lin et al. 2014 do not align with my findings in working with these LNA miR probes. I believe the published results do not depict selective miR staining, but rather ubiquitous staining of DNA and RNA. I believe this because the hybridization temperature at which their FISH experiments were performed is 25˚C, below the DNA melting temperature. The protocol Lin et al. followed is one that has previously been published and shown to produce selective miR staining within the brains of mammals (Silahtaroglu et al. 2007). Silahtaroglu et al. do not share the sequence of the LNA oligonucleotide riboprobes that were used to demonstrate the effectiveness of this technique, so I cannot comment on the validity of their findings. I am confident in my ability to comment on the

findings from Lin et al. 2014 because I used the same sequence and manufacturer to purchase the riboprobes. I also consulted the team of experts that design these probes to get a more thorough understanding of how these probes work and they also agreed that their product would not produce selective miR staining following the published protocol. I believe that if one preformed the FISH experiments looking at the abundance of miR-2954-3p within the NCM across sex and acute song experience following the suggested manufacturer's protocol, it would produce results different from what has been published.

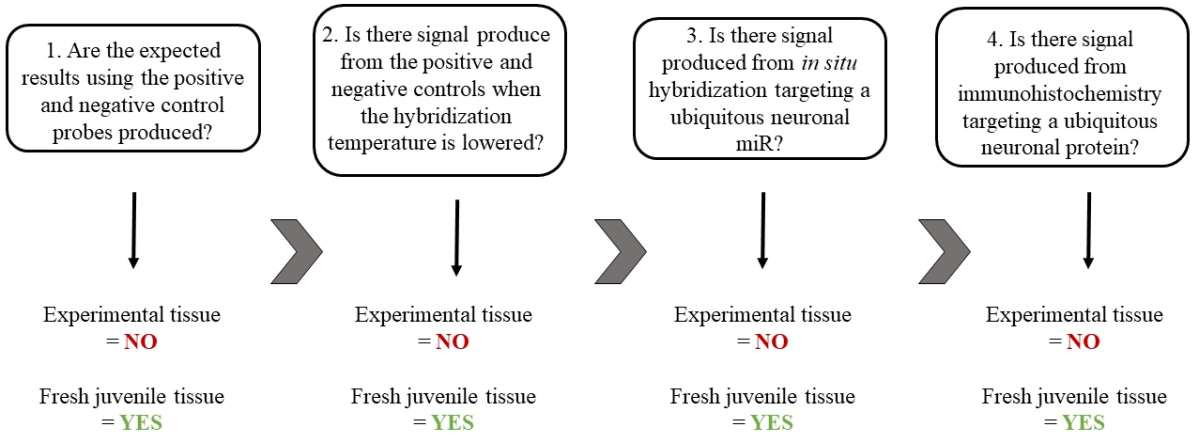

Figure 3-8 Troubleshooting the main ISH experiment with juvenile tissue.
These are the steps that were taken to troubleshoot the main ISH experiment with the juvenile tissue. Each experiment performed to troubleshoot the main ISH experiment is numbered in the order which they were performed. Below each of these are statements is indicated whether or not the numbered experiment produced signal in either the experimental tissue or in freshly collected juvenile tissue.

Table 3-3. Variations on the fixation step.

Percent Paraformaldehyde	Duration on the tissue	Signal on any of the tissue?
3%	3 min	No
3%	5 min	No
3%	10 min	No
4%	3 min	No
4%	5 min	No
4%	10 min	No

This table includes the various iterations of percent paraformaldehyde and duration the tissue was in the fixation solution that were tried to optimize the ISH protocol in a way that resulted in probe signal. The percentage of paraformaldehyde, duration the tissue was within the fixative, and whether or not there was signal produced on these tissues at the end of the ISH experiment are recorded here.

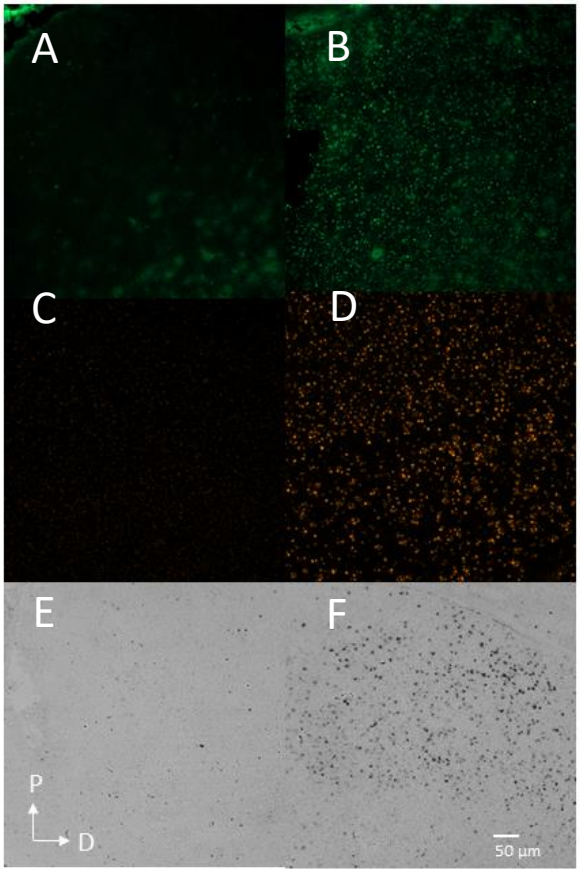

Figure 3-9 Comparison of experimental tissue to new practice tissue using ISH and IHC. This figure includes images from the three experiments that were done to try and salvage the experimental tissue. Panels A, C, and E are images from a P30 male that was collected for the main experiment, in August of 2019. Panels B, D, and F are images from a P30 male that was more recently collected, in December of 2022. Panels A and B are from an ISH with a NR4A3 probe. Panels C and D are from an ISH with a NR1 probe. Panels E and F are from an IHC with NeuN. All images were taken within the NCM, along the dorsal boundary with HP, with the 10X objective. An orientation axis is included in the lower left-hand corner; P = posterior and D = dorsal. A scale bar is included in the lower right-hand corner denoting 50µm.

Chapter IV: Exploring the Abundance of microRNA in Juvenile Males and Females during Developmental Learning Using Real-time PCR

Introduction

After the ISH experiment did not work out, I was still interested in exploring the abundance of miR-2954-3p and miR-2954-5p in the context of juvenile sensory song learning. I had originally intended for the ISH experiment to be accompanied by the data from a real-time PCR experiment, because there is a study investigating this miR within zebra finch auditory forebrain that included the ISH technique, as well as real-time PCR to quantify the changes in miR abundance in adults (Lin et al. 2014). I decided I would continue to build out what the real-time PCR experiment would look like in the form of; predicting potential results, discussing conclusions based off of the possible results, and drawing potential conclusion that could to help inform future studies investigating miR-2954-5p and miR-2954-3p.

Real-time PCR and Gene Expression

Polymerase chain reaction (PCR) is a relatively simple and widely used molecular biology technique to amplify DNA. Real-time -PCR is the ability to monitor the progress of the PCR as it occurs, therefore allowing data to be collected throughout the PCR process, rather than

at the end of the PCR (Holland et al. 1991; Higuchi et al. 1992) **(Figure 4-1).** For some, real-time PCR is considered the most accurate and reliable technique to measure gene expression (Kozera and Rapacz 2013; Bustin et al. 2009). With the addition of a reverse transcription reaction (real-time RT-PCR), real-time PCR, can be used to measure gene expression from mRNA and miR abundance. Real-time RT-PCR is notable for producing high throughput and quantitative results. The disadvantage of real-time RT-PCR is that it destroys the morphology of the sample during RNA extraction. This means that the results produced from real-time RT-PCR will be a measurement of gene expression over the entire section of tissue sampled and will not provide neuroanatomical localization information about the gene expression within the tissue sample.

Real-time RT-PCR as a Complement to ISH in Measuring miR

Real-time RT-PCR can be used in complement with ISH to characterize the expression, abundance, and distribution of miR within a specific tissue. While ISH can provide neuroanatomical spatial information on miR distribution within tissue, real-time RT-PCR complements those results by quantifying the average expression level across the tissue or a specific region of the tissue. Within certain tissues, such as the brain, cell populations are not all alike or uniform. Some cells may have greater expression of certain genes while others may express low levels, or express none at all. Using real-time RT-PCR by itself would not take into account cell-to-cell variation that can be detected by ISH. Together, the results produced with real-time RT-PCR and ISH can not only build a picture of the localization of RNA expression

and the rates of RNA synthesis and degradation within a tissue, cell population or individual cell, but also where in the cell these mechanisms occur.

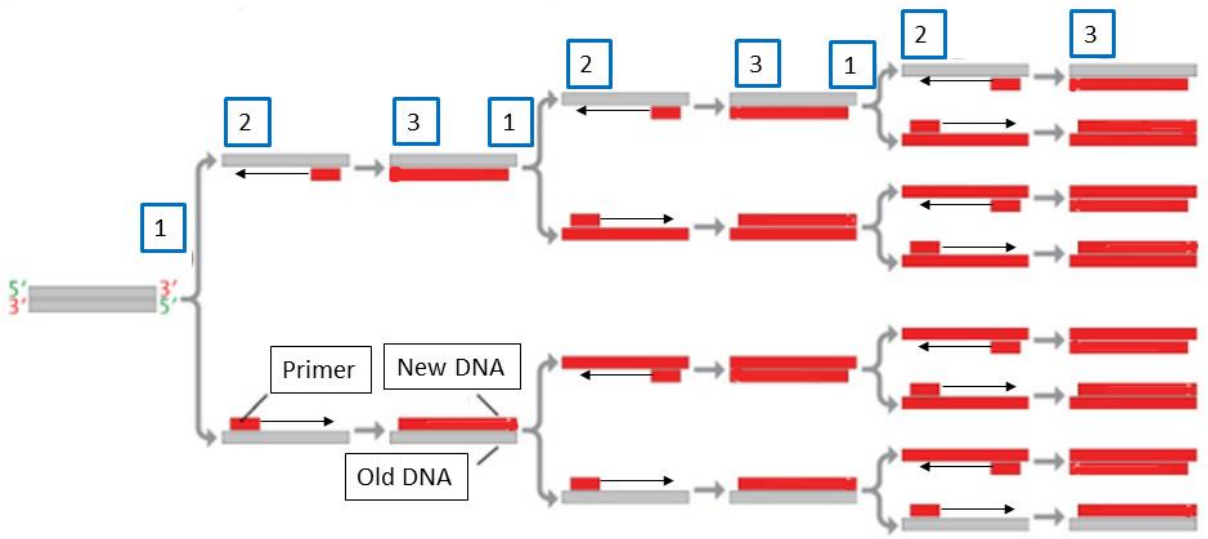

Figure 4-1 Diagram of the real-time RT-PCR method.
After a reverse transcription reaction where mRNA is reverse-transcribed into complementary DNA (cDNA) (the double stranded gray bars on the far left). cDNA is then used as a template for the PCR reaction. During the PCR reaction the cDNA is amplified across several orders of magnitude generating thousands to millions of copies of a particular cDNA sequence. The first step in the PCR is denaturation (labeled here as '1'). The temperature within the PCR machine rises in order to denature the double stranded DNA (dsDNA) into single-stranded DNA. The second step is annealing (labeled here as '2'). The temperature within the PCR machine then decreases to facilitate the binding of primers (labeled 'Primers'; short red bars), or annealing, to the single-stranded DNA (labeled 'Old DNA'; gray bars). The third step is extension (labeled here as '3'). Once primers are annealed, a new strand of DNA is created beginning at the site of the primers (labeled 'New DNA'; long red bars). The process of denaturation, annealing, and extension occurs over and over until the designated number of amplification cycles is achieved. The number of amplification cycles is programmed into the PCR machine for each reaction and can be changed from reaction to reaction. The fluorescent signal used to measure the DNA amplification can be produced from fluorescent reporter molecules in dsDNA binding dye. Each time an amplification cycle is complete, the fluorescent signal increases linearly with the number of fluorescent reporter molecules that bind to dsDNA.

Measuring miR-2954 with ISH and real-time RT-PCR in Zebra Finch

In adult zebra finches, the combination of ISH and real-time RT-PCR have been used to characterize novel miR. Gunaratne et al. 2011 used real-time RT-PCR, along with RNAseq, to investigate novel song responsive miRs in adult auditory forebrain, and they found that miR-2954-3p and miR-2954-5p showed differential changes in abundance in males and females in response to song playbacks (Gunaratne et al. 2011). Building off these findings, Lin et al. 2014 further explored the role of miR-2954-3p via ISH within the brains of adult males and females (Lin et al. 2014). With ISH, they were able to show that miR-2954-3p is present in subsets of cells within the NCM, but did not detect a change in miR-2954-3p abundance in either sex as a result of song playbacks or difference in miR-2954-3p abundance across sex at baseline (Lin et al. 2014). These results from real-time RT-PCR and ISH do not align, but the use of both of these techniques creates an interesting story to investigate because the difference between the two could be due to differences in the number of cells within each of the sub-regions that contain these miR. The tissue used in these real-time RT-PCR experiments included the entire auditory forebrain; NCM, CM, and Field L, while the tissue used for these ISH experiments was just the NCM (Gunaratne et al. 2011; Lin et al. 2014). Thus, miR-2954-3p may be more abundant in the CM and/or Field L compared to the NCM, and this sub-region compartmentalization may contribute to the differences in results between the real-time RT-PCR and ISH experiments in these publications. If this were true, the combination of real-time RT-PCR and ISH results could be used to help further inform the functional differences of the NCM and CM in juvenile sensory song learning across age and sex.

To test if the song responsive changes in miR-2954-3p and miR-2954-5p abundance seen in adults emerge as a function of maturation and experience, I would investigate miR-2954-3p and miR-2954-5p abundance using real-time RT-PCR in juvenile males and females. I would use a song playback paradigm to test miR-2954-5p and miR-2954-3p abundance in response to hearing song (**Figure 4-2**). I would have wanted to test for changes in miR-2954-5p and miR-2954-3p across a known onset and offset of juvenile male sensory song learning. Thus, I would have collected males and females at ages; P23, P30, P60, and P67 (Eales 1985; 1987; Bohner 1983; 1990; Slater et al. 1991; Roper and Zann 2006; Chen et al. 2017; Miller 1979; Clayton 1988; Riebel 2000; 2003; Riebel et al. 2002; Lauay et al. 2004; Terpstra et al. 2004; Holveck and Riebel 2014; Chen et al. 2017; Diez et al. 2021) (**Figure 4-2**). I in order to compliment the ISH I was hoping to have, I would have quantified the abundance of miR-2954-5p and miR-2954-3p within the entire auditory forebrain (**Figure 4-2**). I planned to use the real-time RT-PCR technique to quantify the change in relative abundance of miR-2954-5p and miR-2954-3p after hearing song or being left in silence.

For miR-2954-5p, I would have hypothesized that hearing song would result in no changes in the abundance in the auditory forebrain of juvenile males but would result in a decrease in the abundance in the auditory forebrain of juvenile females. For miR-2954-3p, I would have hypothesized that hearing song would result in an increase in abundance in males and a decrease in abundance in females when both sexes are capable of sensory song learning. My hypotheses are the same as they were for the ISH experiment.

If these predictions were correct, these patterns of miR-2954-5p and miR-2954-3p may indicate that these miR play a part in age- and sex-specific song responses that may contribute to juvenile sensory song learning. Mechanistically, based off previous predicted target analysis, miR-2954-5p and miR-2954-3p are negative regulators of the transcription factor gene NR4A3 (Gunaratne et al. 2011). I did not complete the target analysis using the updated genome assembly, NCBI genome annotation corresponding to the bTaeGut1.4.pri assembly (GCA_003957565.4), to identify additional putative miR-2954-5p and miR-2954-3p targets that may not have been identified with the previous assembly (GCA_000151805.2) (Warren et al. 2010; Rhie et al. 2021). NR4A3 is one of the most strongly song responsive mRNAs in the zebra finch brain and is a predicted target of miR-2954 (Dong et al. 2009; Warren et al. 2010; Lin et al. 2014, Rhie et al. 2021). Inhibition of miR-2954 in a zebra finch cell line produced results that support endogenous miR-2954 expression suppresses MAPK/ERK pathway gene expression (Lin et al. 2014). This is consistent with the mechanistic prediction that miR-2954 is a negative regulator of NR4A3, because a link between ERK signaling and the regulation of NR4A receptor expression has been shown across diverse tissue types and NR4A3 has been shown to be regulated by the MAPK/ERK pathway (Nomiyama et al. 2006; Stocco et al. 2002; Jennings et al. 2020).

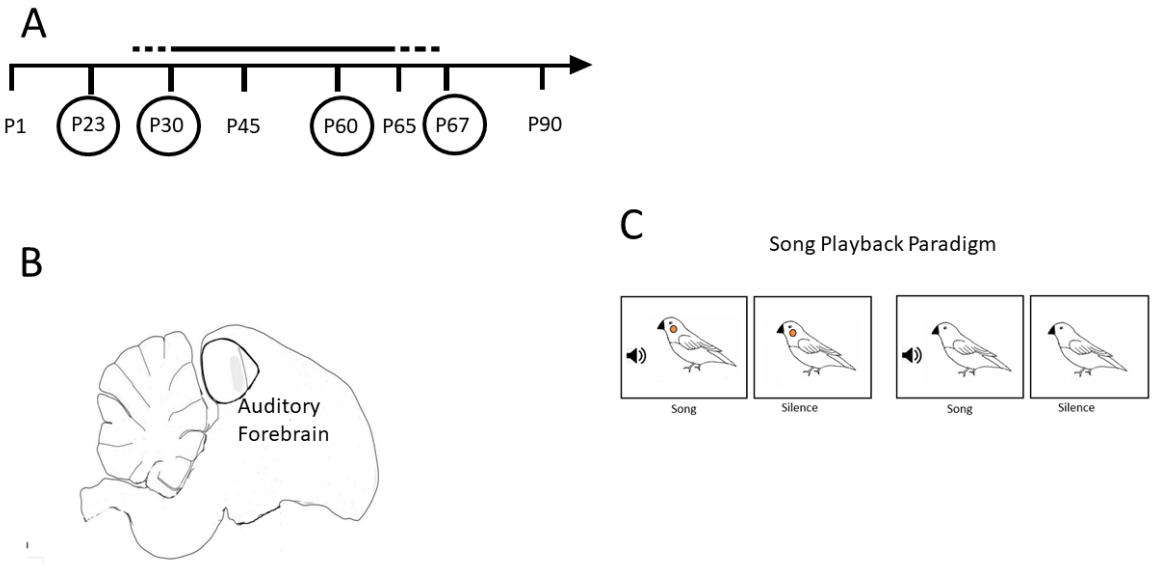

Figure 4-2 Experimental design.
Panel A depicts the ages of the "Normal" juvenile males and females which were collected. Six birds of each sex were collected at each age circled in black. Panel B shows a depiction of the neuroanatomical location of the auditory forebrain, adapted from Figure 3 in London 2019. Panel C shows a depiction of the soundproof chambers the birds were placed in overnight the evening prior to the birds being collected.

Methods

Subjects.

All procedures were conducted in accordance with the NIH guidelines for the care and use of animals for experimentation and were approved by the University of Chicago Institutional Animal Care and Use Committee (ACUP no. 72220).

Rearing.

All zebra finches were housed on a 14 hr:10 hr light:dark cycle, with seed and water provided *ad libitum*. The juveniles used in this study hatched in flight aviaries, amongst males and females of all ages. Juvenile males and females, 24 per sex, were raised in a breeding aviary

and were tutored under normal social conditions (i.e., by their dad or other adult males in the breeding colony). All birds remained in the aviary until the night prior to collection, at which point birds were moved into individual acoustic chambers three hours before lights off to facilitate the song playback paradigm the morning of collection.

Song Playback Paradigm.

All experimental birds were either exposed to a song playback or left in silence. All birds were placed into a soundproof chamber overnight on the evening before they were to be collected. The following morning, two hours after lights come on, those in the song group heard 30 min of a song not heard previously ("Song" condition). Age- and sex-matched controls heard no song playback ("Silence" condition). There were three pairs of Song and Silence condition, n = 3, birds for all age- and sex- combinations (Mello et al. 1992). Birds in the Song condition were exposed to novel conspecific song, "zf101". This song was obtained from a bird bred and raised more than 20 years ago at the Ohio State University by Dr. Susan Volman and were thus novel to all subjects. The acoustic stimuli were played back through a speaker (model FE103, RadioShack) located in the chamber. The average sound intensity was adjusted to 70 dB via a sound pressure meter (model 33-2055, RadioShack).At the end of the song playback or silence period each bird was sacrificed by rapid decapitation. The brain was split in half along the midline using a sterile razor blade. The entire auditory forebrain was removed from each hemisphere and placed in TissueTek O.C.T. Tissue Plus (Fisher HealthCare, cat. no. 4585), and

rapidly frozen on dry ice. At the end of the song playback, all brains were transferred and stored at −80˚C

Real-Time Reverse Transcription PCR (real-time RT-PCR).

RNA samples would have been extracted from zebra finch tissues by TRI Reagent (Ambion), treated with DNase (Ambion). The MicroRNA Assay Kit (Applied Biosystems) would have been used for reverse transcription and real-time RT-PCR of miR-2954 according to manufacturer's instructions as in a previous study (Gunaratne et al. 2011). As an endogenous reference we would have used Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Probe sequences that would have been used for each target miR are given in **Table 4-1**.

Analysis.

The relative gene expression of each individual bird was to be obtained by using the 2^-ΔΔCt method, the same method used by Gunaratne et al. 2011 (Livak and Schmittgen 2001). The 2^-ΔΔCt method consists of a formula which can be used to calculate the relative fold gene expression of samples when performing real-time PCR. Ct stands for the cycle threshold (Ct) of your sample. The Ct value is value which indicates intersection between the threshold and amplification plot. The threshold value is selected for each PCR reaction and is usually set within the exponential phase of PCR. It is the cycle number where the fluorescence generated by the PCR is distinguishable from the background noise. The ΔΔCt is the difference between the ΔCt values of the experimental sample, the Song condition, and the control sample, the Silence condition. The ΔCt is the difference in Ct values for the gene of interest, miR-2954, and the housekeeping gene, GAPDH. Housekeeping genes are important because they serve as a

common denominator to which target gene expression is normalized. Finally, to work out the fold gene expression we need to do 2 to the power of negative ΔΔCt (i.e. the values which have just been created).

Predicted Results

Within the auditory forebrain of juvenile males, I would have expected to see no statistical difference between miR-2954-5p abundance in Song and Silence birds at any of the ages collected; P23, P30, P60, and P67. In juvenile females, I would have expected to see a statistical difference between Song and Silence birds at all ages collected; P23, P30, P60, and P67. The reasoning behind these predicted results is based on the song responsive changes seen in the auditory forebrain of adult males and females measured via real-time PCR (Gunaratne et al. 2011). Both adult males and females perform song learning, in the form of adult song recognition learning, and because of this I would expect hearing song to result in a significant decrease in the relative abundance of any miR involved in song learning. A decrease in the abundance of miR implies there would be an increase in the diversity and abundance of mRNAs available to be translated into proteins. Thus, the significant song response changes in miR-2954-5p abundance in the auditory forebrain of adult females but not in adult males made me think that miR-2954-5p may be a female specific regulator of mRNA available to be translated into proteins that may contribute to the female ability to learn song (Gunaratne et al. 2011).

Within the auditory forebrain of juvenile males, I would have predicted to see a statistical difference between miR-2954-3p abundance in Song and Silence birds at P30 and P60, but not at P23 or P67. In juvenile females, I would have expected to see a statistical difference between

Song and Silence birds at all ages collected; P23, P30, P60 and P67. The reasoning behind these predicted results is based on the song responsive changes seen in the auditory forebrain of adult males and females measured via real-time PCR (Gunaratne et al. 2011). Studies have shown that both adult males and female exhibit the same molecular signatures of song learning (Ahmadiantehrani et al. 2018). Both juvenile males and females perform sensory song learning, but data suggest that juvenile males and females are doing molecularly distinct kinds of sensory song learning in the form of which IEGs are expressed and how the rate of protein synthesis is affected in response to song exposure (Ahmadiantehrani and London 2017; Bailey and Wade 2005; 2003). The song responsive changes in miR-2954-3p abundance were not significant in either adult males or adult females; however, there does appear to be a visual difference between the two (Gunaratne et al. 2011). This visual difference in combination with the fact that molecular signatures of song learning are not the same across sex during development but are the same across sex in adulthood led me to think that maybe miR-2954-3p is a mechanism with song-responsive sex differences during development that do not persist into adulthood. Thus, I would have hypothesized that hearing song will result in a significant increase in the abundance of miR-2954-3p within the NCM and CM of juvenile males and will result in a significant decrease in abundance within the NCM and CM of juvenile females.

Table 4-1. Real-time RT-PCR sequences.

Name of Sequence	Sequence
tgu-miR-124 (F)	5’-GGCACCTGAAATTAAGGC-3’
tgu-miR-124 (R)	5’-CCACTGTAGGCACCATCA-3’
tgu-miR-2954-5p (F)	5’-CCAGCTGGAACGGACTTCTT-3’
tgu-miR-2954-5p (R)	5’-GTGGAGCCTCTCCCTAAACG-3’
tgu-miR-2954-3p (F)	5’-GAACCTTGCCAACTACCCCA-3’
tgu-miR-2954-3p (R)	5’-AGCTCCGCTCCAGATTGTTC-3’
GAPDH (F)	5’-ATGGCATCCAAGGAGTAA-3’
GAPDH (R)	5’-GGAGACAAGGGAACAGAAC-3’
NR4A3 (F)	5’-CTGATCTTCTGCAACGGGGT-3’
NR4A3 (R)	5’-ACCATGGTGTCTGTGTGGTC-3’

This table shows the specific sequence for the forward (“F”) and reverse (“R”) primers I would have used to amplify each gene of interest in real-time RT-PCR. “tgu-” is an indicator that the sequence is specific to the zebra finch genome. GAPDH is the abbreviation for Glyceraldehyde-3-phosphate dehydrogenase, a known house-keeping gene for real-time PCR.

Discussion

When investigating the molecular signatures of sensory song learning in juvenile males and females it is important to analyze potential positive and negative regulators of learning. miR are negative regulators of mRNA available to be translated into proteins that have been shown to play a critical role in learning and memory (Wang et al. 2012; Wang et al. 2021; Aksoy-Aksel et al. 2014; Hu and Li 2017; Thomas et al. 2018). Therefore, to develop a comprehensive understanding of how sensory song learning - and learning in general - is biologically facilitated, it is important to consider the role of miR. More specifically, miR-2954 has the potential to be a

negative regulator of the MAPK/ERK pathway, a well-known pathway in which activation is required in learning and memory (Schafe et al. 2000; Mazzucchelli and Brambilla 2000; Zhen et al. 2001; Kelly et al. 2003; Sharma et al. 2003). Characterizing how song experience affects the abundance of miR-2954 in juvenile males and females will provide further understanding of the similarities/differences in the molecular signatures of juvenile sensory song learning across sex.

The miR-2954-5p results I would have expected in juvenile males would align with the hypothesis that miR-2954-5p may not regulate mRNA that function in facilitating sensory song learning in juvenile males. These results would have mirrored the results seen in adult males after song stimulation (Gunaratne et al. 2011). If the results were to show a statistical difference between Song and Silence birds at P30 and P60, but not at P23 or P67, that would suggest that miR-2954-5p regulates song specific mRNA that may function in sensory song learning. Even though adult males also perform song learning and there is no significant difference between Song and Silence birds in adult males, miR-2954-5p may be regulating the number of mRNA available to be translated into proteins necessary to support sensory song learning because these proteins may be different from those necessary to support adult song recognition learning. (Gunaratne et al. 2011).

The miR-2954-3p results I would have expected in juvenile males would align with the hypothesis that miR-2954-3p may regulate song specific mRNA that may function in juvenile male sensory song learning. If there were significant song responsive changes at any or all of the ages that would have been collected, this would not be consistent with what is known in adults. Prior data show no sex difference in the abundance of miR-2954-3p at baseline or in response to

hearing song within the NCM of adult males (Lin et al. 2014; Gunaratne et al. 2011). The lack of a significant song response in adults could be explained by an attenuated response to hearing song. This could mean that the miR-2954-3p mechanism persists but is just not as robust as it is with less accumulated song experience. The inconsistencies between these potential data and the adult miR-2954-3p data could be due to changes in the baseline levels or the response to song playbacks of miR-2954-3p between juveniles and adults. One way to test this would be to measure the miR-2954-3p abundance levels in juveniles and adults that have not heard song-playbacks (Silence) and compare these abundances to one another. Alternatively, the visual trend in response to song stimulation seen in adults or a significant difference between Song and Silence males may be present beginning at P23. This could suggest that miR-2954-3p does not regulate song specific mRNA or mRNA that potentially function in sensory song learning.

The miR-2954-5p and miR-2954-3p results I would have expected in juvenile females would align with the hypotheses that miR-2954-5p and miR-2954-3p may regulate song specific mRNA that function in facilitating sensory song learning in juvenile females. This miR-2954-5p result would be consistent with adult data, but the miR-2954-3p results would not be consistent with adult data (Gunaratne et al. 2011). Similar to what was discussed in the context of male sensory song learning, the lack of a significant song response in adults could be explained by an attenuated response to hearing song. This would mean that the miR-2954-3p mechanism persists but is just not as robust as it is with less accumulated song experience. Another potential result is significant differences between Song and Silence females at P23 and P67, but not at P30 or P60. This would be an interesting result, because a significant decrease in abundance in Song compared to Silence would support our pS6 findings and the hypothesis that once females have

formed song preferences, they do not learn again until adult song recognition learning. A significant increase in Song compared to Silence could suggest that miR-2954-3p is regulating mRNA available to be translated into proteins that are necessary for sensory song learning, and potentially these proteins could be necessary for juvenile male tutor song memorization. Alternatively, if there was no significant difference in miR-2954-5p/ miR-2954-3p abundance between Song and Silence females at any of the ages that would have been collected or if there was a visual trend in miR-2954-3p abundance in response to song stimulation seen in adults beginning in P23 females, this would suggest miR-2954-5p and/or miR-2954-3p do not regulate song specific mRNA (Gunaratne et al. 2011). Proposed additional experiments are included in the "Conclusions and Future Directions" section.

A decrease in miR-2954-5p and/or miR-2954-3p in response to hearing song implies there would be an increase in the diversity and abundance of mRNAs available to be translated into proteins. An increase in diversity and abundance of mRNAs available to be translated into proteins is consistent with the fact that disrupting new protein synthesis in the auditory forebrain during tutor experiments disrupts tutor song memorization in juvenile males (London and Clayton 2008). In other systems, changing miR abundance leads to a change in the levels of proteins from miR mRNA targets and changes in behavior (Malmevik et al. 2016; Capitano et al. 2015; Busto et al. 2015; Griggs et al. 2013; Vetere et al. 2019; Michely et al. 2015; Sim et al. 2016). Thus, miR-2954-5p and/or miR-2954-3p could be contributing to the changes in

molecular regulation in the female brain in response to hearing song that are required to learn song.

The factors that may be contributing to this change in response after hearing song are age and accumulation of song experience; brain maturation is at different stages between all of the ages I would have collected, and because I would have not removed females from their rearing environment and isolate them from song before I would have collected them, P67 females would have heard more song than P60 than P30 than P23 (Bailey and Wade 2003; Jin and Clayton 1997; Nottebohm and Arnold 1976; Bottjer et al. 1985; Bottjer et al. 1986; Bottjer and Sengelaub 1989; Bottjer et al. 1989). As such, I would not be able to differentiate between the contributions from the underlying maturation and experience-dependent mechanisms.

If the predictions were correct, these results would imply that males and females utilize different neural mechanisms of sensory song learning. There are sex differences in the song processing function of the NCM and CM due to differences in the molecular properties of each (Terpstra et al. 2004; Bolhuis et al. 2000; 2001; Gobes and Bolhuis 2007; Phan et al. 2006; Pinaud et al. 2008; Terpstra et al. 2006, Ahmadiantehrani and London 2017, Bailey and Wade 2003, Bailey and Wade 2005). If there was a sex difference in miR-2954-5p or miR-2954-3p abundance within P23 auditory forebrain this would not be consistent with existing molecular data (Ahmadiantehrani and London 2017). If there was a sex difference in miR-2954-5p or miR-2954-3p abundance within P30 auditory forebrain it would be consistent with other data that show the molecular response to hearing song within the auditory forebrain of P30 males is different than P30 females (Ahmadiantehrani and London 2017; Bailey and Wade 2003;

Stripling et al. 2001). There are no prior molecular or electrophysiological data for P60 and P67 females to be able to make comments on potential sex differences that could be seen at these ages. The pS6+/S6+ cell density findings would suggest there will be a sex difference at both ages but these findings are unable to support any conclusion on the direction of change in miR that would produce a potential sex difference.

Any inconsistencies between these potential data and the adult data could be due to changes in the baseline (Silence) levels of miR-2954-5p or miR-2954-3p throughout development and into adulthood. Silence levels of miR-2954-5p or miR-2954-3p abundance could fluctuate across the ages that would have been collected. Therefore, a sex difference in Silence levels may or may not contribute to the neural maturation that happens in between ages. Depending on if there were any changes in Silence levels, the potential changes in abundance in response to hearing song could suggest that the mechanism that regulates the miR-2954-5p or miR-2954-3p response to hearing song may be the same across sex at certain ages but different at other ages. It has been shown in other systems that there are miRs that are expressed in a sex-specific manner, and that the sex differences in expression emerge at different points during development (Hao and Waxman 2018; Murphy et al. 2014). A higher baseline abundance of miR-2954-5p or miR-2954-3p in adults would make it harder to detect song responsive changes in abundance of miR-2954-5p or miR-2954-3p in adults, if there is an increase in miR abundance after song playbacks, and it is known for a number of other miR that the abundance at baseline changes throughout development (Benoit et al. 2015; Kapsimali et al. 2007; Choi et al. 2008; Sempere et al. 2004). This potential disagreement between the expected juvenile and published adult miR-2954-5p and miR-2954-3p data could also potentially be due to the response to song

playbacks, as adults and juveniles may not transcribe the same genes in response to hearing song. This would be consistent with prior data that show a shift in RNA abundance in response to a stimulus between juveniles and adults (London et al. 2009; Benoit et al. 2015).

Conclusions

If the predictions were true, the results would suggest hearing song leads to distinct changes in molecular mechanism that could influence sensory song learning in juvenile males and females. These experiments would help elucidate what molecular mechanism influences developmental learning in zebra finches.

Chapter V: Conclusions and Future Directions

Summary of Results

The goal of this work was to investigate if the molecular signatures in juvenile male sensory song learning are different from those in juvenile female sensory song learning. I chose to do this by further characterizing the song experience-dependent changes in a molecular indicator of protein synthesis, pS6, and a molecular mechanism that indirectly regulates the protein synthesis of specific mRNA, miR-2954, across age and sex within the auditory forebrain.

Hearing song playbacks resulted in an increase in pS6+/S6+ cell density n juvenile males at P30, P45, and P60, but not at P67 and resulted in an increase in pS6+/S6+ cell density only in P67 females compared to birds left in silence. These results suggest that one indicator of learning within the auditory forebrain of males may be different from that of females during this period of development. More comprehensive studies exploring other molecular markers of learning in females at these ages or studies investigating pS6+/S6+ cell density across a wider age range would need to be done in order to make more definitive conclusions. Other molecular markers to be explored could include all known IEGs and other miR that have been implicated in learning and memory in males and females. These other molecular markers should be explored in males and females at P30, P45, P60, and P67.

I would also investigate pS6+/S6+ cell density across a wider range of ages because behavioral data show that females are learning song from ~P25 – P70 (Miller 1979; Clayton 1990; Riebel 2000; Riebel et al. 2002, Clayton 1988). I would begin by expanding the ages which were tested to include P10 - P30 birds. We know that Normal males and females form song preferences between P25 - P35 (Clayton 1988). If I fail to reject my hypothesis about females having

established song preference by P30 and not learning again until adult song recognition learning then I would predict that performing this experiment again including collecting birds as early as P10, would provide the data to show a period of pS6+/S6+ song response occurring between P10 and up until some age between P25 and P30.

It would also be interesting to compare the pS6+/S6+ cell density results in P10 - P30 juvenile females to juvenile males at these ages. Acute song playbacks do not produce a pS6+/S6+ song response in P23 males; if this lack of a pS6+/S6+ song response was consistent from P10 - P30, that could suggest that the type of song learning to support song preference in juvenile males is different from juvenile male tutor song memorization learning (Ahmadiantehrani and London 2017; Clayton 1988). If the acute song playbacks do not produce a pS6+/S6+ song response in either juvenile males or females prior to P30, this would be contradictory to existing behavioral data that show juvenile males and females are capable of forming lasting memories of their father's song around this age, between P25 - P35 (Clayton 1988). Even if a pS6+/S6+ song response is found, this does not necessarily mean the bird is learning. pS6 is necessary for the activity of the ribosome, but that does not mean that the molecular mechanisms downstream of protein synthesis necessary to support learning have been established. The lack of a significant pS6+/S6+ song response also does not necessarily mean that the bird is not learning. It is unknown what proportion of cells within the auditory forebrain are needed to have experience-dependent new protein synthesis to support learning. I do not know what the minimal amount of cells would be or of any evidence to support this claim, but I predict it would be unlikely that no cells within the auditory forebrain have pS6 and the bird is still learning, because the ribosome must be active for protein synthesis to occur. In order to determine what proportion of cells within the auditory forebrain is

needed to have experience-dependent new protein synthesis, one would need to be able to selectively inhibit the phosphorylation of S6 in individual cells.

The miR-2954-5p and miR-2954-3p work would have potentially revealed song responsive changes in miR-2954-5p and miR-2954-3p abundance that would suggest that the products of the miR-2954 gene are potential regulators of sensory song learning. If acute song experience resulted in the same directional regulation of miR-2954-5p/miR-2954-3p in both males and females between P30 - P67, it wouldn't necessarily mean that females are capable of memorizing tutor song like juvenile males do. Instead, it would suggest that females possess one the molecular mechanisms that may support tutor song memorization learning. Like I mentioned previously, if there is no significant difference in the relative abundance of miR-2954-5p or miR-2954-5p between Song and Silence birds that does not necessarily mean that there is no learning happening or that miR-2954 does not regulate the formation of proteins necessary to support sensory song learning. As with pS6, it is unknown what proportion of cells within the auditory forebrain are needed to have experience-dependent new protein synthesis to support learning.

The miR-2954 work could have also produced results that may have shed more light on the pS6+/S6+ findings. The miR work included and would have included a collection at P23, which could support the hypothesis that juvenile females establish preference for songs prior by P30 and then do not learn song again until adult song recognition learning. Seeing a song responsive decrease in either miR-2954-5p or miR-2954-3p at P23 and P67, but not at the ages in between, would support this hypothesis.

The common thread among the projects is an experimental design that would produce results which could be used to characterize the molecular signatures of juvenile sensory song learning in each sex. In the field of zebra finch research, there are wide gaps in knowledge of what is known about sensory song learning compared to motor learning in males, sensory song learning in males compared to sensory song learning in females, and how sensory song learning is biologically facilitated in females. My dissertation work has contributed to narrowing these gaps by investigating both sexes. Some of my work also has the potential to produce results that could contribute to medical advancements. miR-2954 has the potential to be a negative regulator of the MAPK/ERK pathway, a well-known pathway in which activation is required in learning and memory. The MAPK pathway has also been extensively studied in the context of oncogenesis. The dysregulation of the MAPK pathway is one of the primary causes of cancer. While characterizing how song experience affects the abundance of miR-2954 doesn't have a direct relationship to cancer research, further characterizing a potential negative regulator of the MAPK pathway could provide valuable information in forming a new therapeutic pathway for cancer patients.

Future Directions

Further exploring pS6+/S6+ cell density in juvenile female song learning

As has been stated previously, more is known about the mechanisms which influence sensory song learning in juvenile males compared to juvenile females. The pS6 experiment produced particularly interesting results in females. The emergence of a pS6+/S6+ song response at P67, but not at any age prior, does not align with the sensory song learning window in juvenile

males. Could this result be indicative of a "delayed" sensory song learning window? Or could it be indicative of formation of mechanisms needed to support adult song recognition learning? One way to further contextualize this result would be to determine if pS6 is necessary for juvenile and/or adult song learning. One way of doing this is to bidirectionally manipulated the mTOR cascade activation *in vivo*, via constitutively activating and selectively inhibiting mTOR signaling in females between P25 and P70. There is behavioral data that suggest females are sensory song learning during within this window of development (Miller 1979; Clayton 1990; Riebel 2000; Riebel et al. 2002, Clayton 1988). You cannot selectively inhibit the phosphorylation of S6, but you can selectively manipulate mTOR signaling. pS6 is a functional readout of mTOR cascade activation because the S6 kinase is itself directly phosphorylated by mTOR complex 1 kinase activity (Hay and Sonenberg 2004; Magnuson et al. 2012). The bidirectional manipulation of mTOR was used previously to demonstrate that mTOR signaling is required for tutor song memorization in juvenile males (Ahmadiantehrani and London 2017). In order to do this in females, because females do not sing, you would need to utilize a behavioral paradigm that can act as a proxy for sensory song learning. I would do this by using the operant preference learning paradigm developed by Riebel 2000 and combining it with the experimental paradigm developed in Ahmadiantehrani and London 2017. The operant preference learning paradigm supports the establishment of song preference when molecular mechanisms are not disrupted (Riebel 2000). The experimental paradigm created by Ahmadiantehrani and London 2017 includes infusion time-course experiments with drugs; one that constitutively activate and another that selectively inhibit mTOR signaling into the auditory forebrain. I would do these infusion experiments before initiating the operant preference learning paradigm. You would need to perform these time-course experiments in order to determine when to infuse each drug prior to the preference learning

paradigm to ensure the drugs would be effective throughout the entire paradigm. These time-course experiments have been done in juvenile males in the context of tutor song memorization and these experiments can be used to inform what the appropriate drug infusion may be in the context of female song preference learning (Ahmadiantehrani and London 2017). Once you have established this sensory song learning paradigm and determined the appropriate time to infuse the drugs prior to the paradigm, you would bidirectionally manipulate mTOR signaling *in vivo* in females for the duration of the paradigm and compare how these females perform on song preference tests compared to control females who did not receive drug infusions. Currently, these experiments are being performed in the London Lab.

If the mechanisms of juvenile female sensory song learning which support the establishment of song preference are the same as the mechanisms of tutor song memorization in juvenile males, then I would predict that both constitutively activating and selectively inhibiting mTOR signaling would abolish the ability for females to establish song preference. If constitutively activating and selectively inhibiting mTOR signaling does not prevent the establishment of song preference, then this would be another piece of evidence that suggests the mechanisms underlying sensory song learning in juvenile females are distinct from those of juvenile male sensory song learning. Additionally, the results from this experiment would produce data to help characterize the role of mTOR and potentially pS6 within the NCM and CM of juvenile female sensory song learning.

How age and accumulated song experience influence the song responsive changes in miR-2954-5p and miR-2954-3p

One of the key factors that can influence learning that would not have been tested for in the miR-2954-5p and miR-2954-3p experiments, is the accumulation of experience. A logical next follow-up study to these proposed experiments would be to control for the accumulation of song experience by having Tutored and Isolate males and females that could be collected at each of the proposed ages; P23, P30, P60, and P67. Including both males and females would continue to allow me to draw conclusions about the similarities and differences between juvenile male and female sensory song learning. I would proposed to collect Tutors and Isolates at each of these ages, because these ages span the onset and offset of sensory song learning in males and span across the ages that are considered to be included within the window of juvenile female sensory song learning (Eales 1985; 1987; Bohner 1983; Slater et al. 1991; Roper and Zann 2006; Chen et al. 2017; Miller 1979; Clayton 1988; Riebel 2000; Riebel et al. 2002; Lauay et al. 2004; Terpstra et al. 2006; Holveck and Riebel 2014; Diez et al. 2021).

The results from this experiment could help contextualize the potential role miR-2954-5p and miR-2954-3p have in regulating sensory song learning ability. If miR-2954-5p and/or miR-2954-3p regulate the translation of mRNA necessary for tutor song memorization, then I would expect that the abundance of miR-2954-5p and miR-2954-3p in Tutored Song males at P30 and P60 would be significantly lower than the abundance of these miR at P23 and P67 in Tutored Silence males. A decrease in miR-2954-5p and/or miR-2954-3p in response to hearing song implies there would be an increase in the diversity and abundance of mRNAs available to be translated into proteins. An increase in diversity and abundance of mRNAs available to be translated into proteins is consistent with the fact that presumably disrupting new protein synthesis by constitutive activation of mTOR in the auditory forebrain of juvenile males during tutor experiments disrupts tutor song memorization (Ahmadiantehrani and London 2017). Constitutive

activation of mTOR would lead to an increased number of cells with active ribosomes, via the phosphorylation of S6, and an increase in the number of active ribosomes could result in an increased number of mRNA being translated into proteins. In Tutor females, I would expect that the abundance of miR-2954-5p and miR-2954-3p would be significantly lower in Song females compared to Silence females at P23 and P67, but not at P30 and P60. This prediction is in support of the hypothesis I made based off the pS6+/S6+ results. In Isolates, I would predict that both male and female Song birds will have significantly lower abundance in miR-2954-5p and miR-2954-3p compared to Silence birds, but not at the same ages across sex. In Isolate males, I would expect to see a significant decrease in miR abundance in Song compared to Silence males at P30 and P60, but not at P23 or P67. This is because prior data show that P30 Isolate Song males have a more robust increase in the pS6+/S6+ compared to P30 Isolate Silence males and that tutor song experience prior to P30 does not support tutor song memorization, but it does influences neural properties important for tutor song memorization (Braaten 2010; Adret et al. 2012; Chen et al. 2017; Ahmadiantehrani and London 2017; Roper and Zann 2006). In Isolate females, I would expect to see a significant decrease in miR abundance in Song compared to Silence females across all of the ages collected; P23, P30, P60, and P67. In support of my hypothesis about the establishment of female song preference, Isolate females will not have had sufficient song experience in order to establish preference by any of these ages so I would expect to see a significant song response at each of these ages. P23 and P30 Normal females do not show a significant increase in pS6+/S6+ cell density in Song compared to Silence birds, there is no preexisting data on P30 Isolate females (Ahmadiantehrani and London 2017). This lack of significant pS6+/S6+ in response to song may be due to the prior accumulation of song experience, and if it is, then this would support the predictions I made about Isolate females.

Comparing molecular mechanisms of sensory song learning in masculinized females to those in genetic males and females

One way to assess the similarities and differences in sensory song learning ability between males and females is to create females who can sing and then compare the molecular profiles of these animals to those of males and unmanipulated females. It has been demonstrated that you can create females who sing via estradiol administration during development, which masculinizes their brain nuclei (Gurney 1982, Gurney and Konishi 1980, Simpson and Vicario 1991, Adkins-Regan et al. 1994). The song that estradiol treated females sing in adulthood is learned during development (Simpson and Vicario 1991). The hypothesis would be that in females with masculinized brain nuclei, song responsive molecular mechanisms of song learning and anatomical connections between the brain regions necessary for tutor song memorization and song production will look more similar to males than to hormonally unmanipulated females. Comparing how hearing song affects pS6 and miR-2954-5p/ miR-2954-3p across these three groups could provide information on whether or not juvenile females are capable of memorizing song in the same way as juvenile males. This study would also provide information on the differences in number of cell types and cellular organization within the auditory forebrain in juvenile male and female song learning.

Supplementary Materials:

Table S1 Three-way ANOVA output from the Normal NCM measurements.

3-Way ANOVA					
	Df	Sum Sq	Mean Sq	F	Pr(>F)
AGE	3	5.262	1.7539	6.937	0.000999
SEX	1	2.245	2.2451	9.174	0.004826
CONDITION	1	1.541	1.5413	10.269	0.00306
AGE:SEX	3	4.951	1.6504	6.48	0.001495
AGE:CONDITION	3	0.764	0.2545	2.229	0.10383
SEX:CONDITION	1	1.329	1.3287	9.015	0.005162
AGE:SEX:CONDITION	3	0.223	0.0742	0.894	0.454797
Residuals	32	8.199	0.2562		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Tukey multiple comparisons of means

Tukey multiple comparison of means

95% family-wise confidence level

Fit: aov(formula = BASELINE ~ SEX + CONDITION + AGE + AGE * SEX + AGE * CONDITION + SEX * CONDITION, data = d)

$SEX				
	diff	lwr	upr	p adj
M-F	0.432545	0.13491	0.730179	0.004826
$CONDITION				
	diff	lwr	upr	p adj
Song-Sil	0.358389	0.060754	0.656023	0.00306
$AGE				
	diff	lwr	upr	p adj
P45-P30	-0.78645	-1.34632	-0.22658	0.00289
P60-P30	-0.72903	-1.2889	-0.16916	0.006728
P67-P30	-0.77359	-1.33347	-0.21372	0.0038
P60-P45	0.057416	-0.50246	0.617287	0.992361
P67-P45	0.012854	-0.54702	0.572725	0.999912
P67-P60	-0.04456	-0.60443	0.51531	0.996384
$`SEX:AGE`				
	diff	lwr	upr	p adj
P45:F-P30:F	-0.01339	-0.96003	0.93326	1

P60:F-P30:F	0.031105	-0.91554	0.97775	1
P67:F-P30:F	-0.09624	-1.04288	0.850408	0.999975
P30:M-P30:F	1.537822	0.591177	2.484468	0.000226
P45:M-P30:F	-0.02169	-0.96833	0.924958	1
P60:M-P30:F	0.048654	-0.89799	0.995299	1
P67:M-P30:F	0.086872	-0.85977	1.033517	0.999987
P60:F-P45:F	0.04449	-0.90216	0.991135	1
P67:F-P45:F	-0.08285	-1.0295	0.863793	0.999991
P30:M-P45:F	1.551208	0.604562	2.497853	0.000199
P45:M-P45:F	-0.0083	-0.95495	0.938343	1
P60:M-P45:F	0.062039	-0.88461	1.008685	0.999999
P67:M-P45:F	0.100257	-0.84639	1.046903	0.999966
P67:F-P60:F	-0.12734	-1.07399	0.819303	0.999832
P30:M-P60:F	1.506718	0.560072	2.453363	0.000306
P45:M-P60:F	-0.05279	-0.99944	0.893853	1
P60:M-P60:F	0.017549	-0.9291	0.964195	1
P67:M-P60:F	0.055767	-0.89088	1.002413	0.999999
P30:M-P67:F	1.63406	0.687414	2.580705	8.84E-05
P45:M-P67:F	0.07455	-0.8721	1.021195	0.999996
P60:M-P67:F	0.144891	-0.80175	1.091537	0.999604
P67:M-P67:F	0.183109	-0.76354	1.129755	0.998203
P45:M-P30:M	-1.55951	-2.50616	-0.61286	0.000183
P60:M-P30:M	-1.48917	-2.43581	-0.54252	0.000363
P67:M-P30:M	-1.45095	-2.3976	-0.50431	0.000526
P60:M-P45:M	0.070341	-0.8763	1.016987	0.999997
P67:M-P45:M	0.108559	-0.83809	1.055205	0.999942
P67:M-P60:M	0.038218	-0.90843	0.984864	1
$`CONDITION:AGE`				
	diff	lwr	upr	p adj
P45:Sil-P30:Sil	-0.56827	-1.51492	0.378373	0.532413
P60:Sil-P30:Sil	-0.37561	-1.32226	0.571032	0.897648
P67:Sil-P30:Sil	-0.57646	-1.5231	0.37019	0.514712
P30:Song-P30:Sil	0.742754	-0.20389	1.6894	0.214927
P45:Song-P30:Sil	-0.26187	-1.20851	0.684777	0.984323
P60:Song-P30:Sil	-0.3397	-1.28634	0.606949	0.936733
P67:Song-P30:Sil	-0.22798	-1.17462	0.718667	0.993041
P60:Sil-P45:Sil	0.192659	-0.75399	1.139304	0.997524

P67:Sil-P45:Sil	-0.00818	-0.95483	0.938463	1
P30:Song-P45:Sil	1.311027	0.364382	2.257672	0.002004
P45:Song-P45:Sil	0.306405	-0.64024	1.25305	0.962747
P60:Song-P45:Sil	0.228577	-0.71807	1.175222	0.99293
P67:Song-P45:Sil	0.340295	-0.60635	1.28694	0.936178
P67:Sil-P60:Sil	-0.20084	-1.14749	0.745804	0.996791
P30:Song-P60:Sil	1.118368	0.171723	2.065013	0.011745
P45:Song-P60:Sil	0.113746	-0.8329	1.060391	0.999921
P60:Song-P60:Sil	0.035918	-0.91073	0.982563	1
P67:Song-P60:Sil	0.147636	-0.79901	1.094281	0.999553
P30:Song-P67:Sil	1.31921	0.372564	2.265855	0.001855
P45:Song-P67:Sil	0.314587	-0.63206	1.261233	0.957212
P60:Song-P67:Sil	0.23676	-0.70989	1.183405	0.991281
P67:Song-P67:Sil	0.348478	-0.59817	1.295123	0.928265
P45:Song-P30:Song	-1.00462	-1.95127	-0.05798	0.03121
P60:Song-P30:Song	-1.08245	-2.0291	-0.1358	0.016098
P67:Song-P30:Song	-0.97073	-1.91738	-0.02409	0.041212
P60:Song-P45:Song	-0.07783	-1.02447	0.868818	0.999994
P67:Song-P45:Song	0.03389	-0.91276	0.980536	1
P67:Song-P60:Song	0.111718	-0.83493	1.058363	0.99993
$`SEX:CONDITION`				
	diff	lwr	upr	p adj
M:Sil-F:Sil	0.09979	-0.46008	0.659662	0.962339
F:Song-F:Sil	0.025634	-0.53424	0.585506	0.999303
M:Song-F:Sil	0.790933	0.231062	1.350805	0.000609
F:Song-M:Sil	-0.07416	-0.63403	0.485715	0.983883
M:Song-M:Sil	0.691143	0.131272	1.251015	0.000642
M:Song-F:Song	0.765299	0.205428	1.325171	0.00091

This table shows the three-way ANOVA output from the Normal NCM pS6+/S6+ cell density measurements from Chapter 3. The p-values highlighted in yellow are the significant p-values, $p < 0.05$.

Table S2 Three-way ANOVA output from the Normal CM measurements.

3-Way ANOVA					
	Df	Sum Sq	Mean Sq	F	Pr(>F)
SEX	1	1.237	1.237	1.806	0.18768
CONDITION	1	7.383	7.383	10.775	0.00234
AGE	3	5.708	1.903	2.777	0.05563
SEX:AGE	3	6.516	2.172	3.17	0.03626
CONDITION:AGE	3	1.651	0.55	0.803	0.50065
SEX:CONDITION	1	7.781	7.781	11.356	0.00184
Residuals	35	23.983	0.685		
SEX:CONDITION:AGE	3	1.048	0.349	0.487	0.6934

Tukey multiple comparisons of means

Tukey multiple comparisons of means

95% family-wise confidence level

Fit: aov(formula = BASELINE ~ SEX + CONDITION + AGE + SEX * AGE + CONDITION * AGE + SEX * CONDITION, data = cm)

$SEX				
	diff	lwr	upr	p adj
M-F	0.321101	-0.16401	0.806214	0.187678
$CONDITION				
	diff	lwr	upr	p adj
Song-Sil	0.784395	0.299281	1.269508	0.002338
$AGE				
	diff	lwr	upr	p adj
P45-P30	-0.54787	-1.45926	0.36352	0.380239
P60-P30	-0.62639	-1.53778	0.284997	0.266338
P67-P30	-0.96036	-1.87175	-0.04897	0.035695
P60-P45	-0.07852	-0.98991	0.832868	0.995498
P67-P45	-0.41249	-1.32388	0.498904	0.618217
P67-P60	-0.33396	-1.24536	0.577427	0.757019
$`SEX:AGE`				
	diff	lwr	upr	p adj
M:P30-F:P30	1.544835	0.005511	3.084159	0.048651
F:P45-F:P30	0.432729	-1.10659	1.972053	0.98356
M:P45-F:P30	0.016364	-1.52296	1.555688	1
F:P60-F:P30	0.067725	-1.4716	1.607048	1
M:P60-F:P30	0.224323	-1.315	1.763646	0.999729
F:P67-F:P30	-0.18761	-1.72693	1.351716	0.999918
M:P67-F:P30	-0.18827	-1.7276	1.351051	0.999916

F:P45-M:P30	-1.11211	-2.65143	0.427218	0.30826
M:P45-M:P30	-1.52847	-3.06779	0.010853	0.052754
F:P60-M:P30	-1.47711	-3.01643	0.062213	0.06769
M:P60-M:P30	-1.32051	-2.85984	0.218812	0.137735
F:P67-M:P30	-1.73244	-3.27177	-0.19312	0.018359
M:P67-M:P30	-1.73311	-3.27243	-0.19378	0.018293
M:P45-F:P45	-0.41637	-1.95569	1.122958	0.986826
F:P60-F:P45	-0.365	-1.90433	1.174319	0.99396
M:P60-F:P45	-0.20841	-1.74773	1.330917	0.999834
F:P67-F:P45	-0.62034	-2.15966	0.918987	0.893497
M:P67-F:P45	-0.621	-2.16033	0.918321	0.892973
F:P60-M:P45	0.051361	-1.48796	1.590684	1
M:P60-M:P45	0.207959	-1.33136	1.747283	0.999837
F:P67-M:P45	-0.20397	-1.7433	1.335352	0.999856
M:P67-M:P45	-0.20464	-1.74396	1.334687	0.999853
M:P60-F:P60	0.156598	-1.38273	1.695922	0.999976
F:P67-F:P60	-0.25533	-1.79466	1.283991	0.999366
M:P67-F:P60	-0.256	-1.79532	1.283326	0.999355
F:P67-M:P60	-0.41193	-1.95125	1.127393	0.987619
M:P67-M:P60	-0.4126	-1.95192	1.126728	0.987502
M:P67-F:P67	-0.00067	-1.53999	1.538659	1
$`CONDITION:AGE`				
	diff	lwr	upr	p adj
Song:P30-Sil:P30	1.348454	-0.19087	2.887777	0.122062
Sil:P45-Sil:P30	-0.24313	-1.78245	1.296193	0.99954
Song:P45-Sil:P30	0.495842	-1.04348	2.035166	0.965104
Sil:P60-Sil:P30	-0.10543	-1.64475	1.433898	0.999998
Song:P60-Sil:P30	0.201092	-1.33823	1.740415	0.999869
Sil:P67-Sil:P30	-0.65795	-2.19727	0.881375	0.861511
Song:P67-Sil:P30	0.085686	-1.45364	1.62501	1
Sil:P45-Song:P30	-1.59158	-3.13091	-0.05226	0.038458
Song:P45-Song:P30	-0.85261	-2.39194	0.686712	0.634394
Sil:P60-Song:P30	-1.45388	-2.9932	0.085445	0.075582
Song:P60-Song:P30	-1.14736	-2.68669	0.391962	0.272449
Sil:P67-Song:P30	-2.0064	-3.54573	-0.46708	0.003922
Song:P67-Song:P30	-1.26277	-2.80209	0.276556	0.175209
Song:P45-Sil:P45	0.738973	-0.80035	2.278296	0.777428
Sil:P60-Sil:P45	0.137705	-1.40162	1.677029	0.99999
Song:P60-Sil:P45	0.444222	-1.0951	1.983546	0.980926
Sil:P67-Sil:P45	-0.41482	-1.95414	1.124506	0.987107
Song:P67-Sil:P45	0.328816	-1.21051	1.86814	0.996817
Sil:P60-Song:P45	-0.60127	-2.14059	0.938056	0.907863

Song:P60-Song:P45	-0.29475	-1.83407	1.244573	0.998401
Sil:P67-Song:P45	-1.15379	-2.69311	0.385533	0.266224
Song:P67-Song:P45	-0.41016	-1.94948	1.129167	0.987926
Song:P60-Sil:P60	0.306517	-1.23281	1.845841	0.997951
Sil:P67-Sil:P60	-0.55252	-2.09185	0.986801	0.938881
Song:P67-Sil:P60	0.191111	-1.34821	1.730435	0.999907
Sil:P67-Song:P60	-0.85904	-2.39836	0.680284	0.625817
Song:P67-Song:P60	-0.11541	-1.65473	1.423918	0.999997
Song:P67-Sil:P67	0.743634	-0.79569	2.282958	0.772037
$`SEX:CONDITION`				
	diff	lwr	upr	p adj
M:Sil-F:Sil	-0.48415	-1.39554	0.427244	0.488116
F:Song-F:Sil	-0.02085	-0.93224	0.890538	0.999914
M:Song-F:Sil	1.105495	0.194104	2.016886	0.012308
F:Song-M:Sil	0.463294	-0.4481	1.374685	0.525462
M:Song-M:Sil	1.589642	0.678251	2.501033	0.000221
M:Song-F:Song	1.126348	0.214957	2.037739	0.010487

This table shows the three-way ANOVA output from the Normal CM pS6+/S6+ cell density measurements from Chapter 3. The p-values highlighted in yellow are the significant p-values, $p < 0.05$ and the p-values highlighted in peach are p-values that are either slightly above or below $p < 0.05$.

Table S3 Three-way ANOVA output from the P67 male NCM measurements.

2-Way ANOVA					
	Df	Sum Sq	Mean Sq	F	Pr(>F)
CONDITION (norm/tut/iso)	2	0.3304	0.1652	1.185	0.33912
EXPERIENCE (song/sil)	1	1.3765	1.3765	9.873	0.0085
CONDITION:EXPERIENCE	2	2.0932	1.0466	7.507	0.00768
Residuals	12	1.673	0.1394		

Tukey multiple comparisons of means

Tukey multiple comparison of means

95% family-wise confidence level

Fit: aov(formula = BASELINE ~ CONDITION + EXPERIENCE + CONDITION*EXPERIENCE, data = data_P67_ncm)

$CONDITION				
	diff	lwr	upr	p adj
Norm-Iso	-0.3224652	-0.89759	0.252663	0.327155
Tut-Iso	-0.2291821	-0.80431	0.345947	0.553531
Tut-Norm	0.0932831	-0.48185	0.668412	0.902752
$EXPERIENCE				
	diff	lwr	upr	p adj
Song-Sil	0.5530795	0.16957	0.936589	0.008497
$`CONDITION:EXPERIENCE`				
	diff	lwr	upr	p-adjusted
Norm:Sil-Iso:Sil	0.09688836	-0.92715	1.120927	0.999444
Tut:Sil-Iso:Sil	0.60612224	-0.41792	1.63016	0.401346
Iso:Song-Iso:Sil	1.38951803	0.36548	2.413556	0.006668
Norm:Song-Iso:Sil	0.64769931	-0.37634	1.671737	0.337217
Tut:Song-Iso:Sil	0.32503163	-0.69901	1.34907	0.88558
Tut:Sil-Norm:Sil	0.50923387	-0.5148	1.533272	0.573074
Iso:Song-Norm:Sil	1.29262966	0.268592	2.316668	0.011324
Norm:Song-Norm:Sil	0.55081095	-0.47323	1.574849	0.496422
Tut:Song-Norm:Sil	0.22814327	-0.79589	1.252181	0.971291
Iso:Song-Tut:Sil	0.78339579	-0.24064	1.807434	0.178721
Norm:Song-Tut:Sil	0.04157707	-0.98246	1.065615	0.999991
Tut:Song-Tut:Sil	-0.2810906	-1.30513	0.742948	0.933242
Norm:Song-Iso:Song	-0.74181872	-1.76586	0.282219	0.219104

Tut:Song-Iso:Song	-1.06448639	-2.08852	-0.04045	0.040014
Tut:Song-Norm:Song	-0.32266768	-1.34671	0.70137	0.888513
Iso:Song-Iso:Sil	1.38951803	0.36548	2.413556	0.006668
Iso:Song-Norm:Sil	1.29262966	0.268592	2.316668	0.011324
Tut:Song-Iso:Song	-1.06448639	-2.08852	-0.04045	0.040014

This table shows the three-way ANOVA output from the P67 male NCM pS6+/S6+ cell density measurements from Chapter 3. The p-values highlighted in yellow are the significant p-values, $p < 0.05$.

Table S4 Three-way ANOVA output from the P67 male CM measurements.

2-Way ANOVA					
	Df	Sum Sq	Mean Sq	F	Pr(>F)
CONDITION	2	0.009	0.0046	0.01	0.9897
EXPERIENCE	1	2.121	2.1209	4.77	0.0495
CONDITION:EXPERIENCE	2	2.674	1.337	3.007	0.0874
Residuals	12	5.336	0.4446		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Tukey multiple comparisons of means

Tukey multiple comparisons of means

95% family-wise confidence level

Fit: aov(formula = BASELINE ~ CONDITION + EXPERIENCE + CONDITION * EXPERIENCE, data = data_P67)

$CONDITION				
	diff	lwr	upr	p adj
Norm-Iso	-0.0484	-1.0755	0.978696	0.99133
Tut-Iso	-0.00075	-1.02785	1.026347	0.999998
Tut-Norm	0.047651	-0.97945	1.074749	0.991596
$EXPERIENCE				
	diff	lwr	upr	p adj
Song-Sil	0.686522	0.001629	1.371415	0.049538
$`CONDITION:EXPERIENCE`				
	diff	lwr	upr	p adj
Norm:Sil-Iso:Sil	-0.31578	-2.14457	1.513005	0.990565
Tut:Sil-Iso:Sil	0.649708	-1.17908	2.478494	0.831938
Iso:Song-Iso:Sil	0.941909	-0.88688	2.770695	0.539161
Norm:Song-Iso:Sil	1.160886	-0.6679	2.989672	0.333812
Tut:Song-Iso:Sil	0.290699	-1.53809	2.119485	0.993521
Tut:Sil-Norm:Sil	0.965489	-0.8633	2.794276	0.514885
Iso:Song-Norm:Sil	1.25769	-0.5711	3.086476	0.261687
Norm:Song-Norm:Sil	1.476667	-0.35212	3.305453	0.143454
Tut:Song-Norm:Sil	0.60648	-1.22231	2.435267	0.866617
Iso:Song-Tut:Sil	0.292201	-1.53659	2.120987	0.993367
Norm:Song-Tut:Sil	0.511178	-1.31761	2.339964	0.928422
Tut:Song-Tut:Sil	-0.35901	-2.1878	1.469777	0.983323
Norm:Song-Iso:Song	0.218977	-1.60981	2.047763	0.998276
Tut:Song-Iso:Song	-0.65121	-2.48	1.177577	0.830666

Tut:Song-Norm:Song	-0.87019	-2.69897	0.9586	0.614436

This table shows the three-way ANOVA output from the P67 male CM pS6+/S6+ cell density measurements from Chapter 3. The p-values highlighted in yellow are the significant p-values, $p < 0.05$.

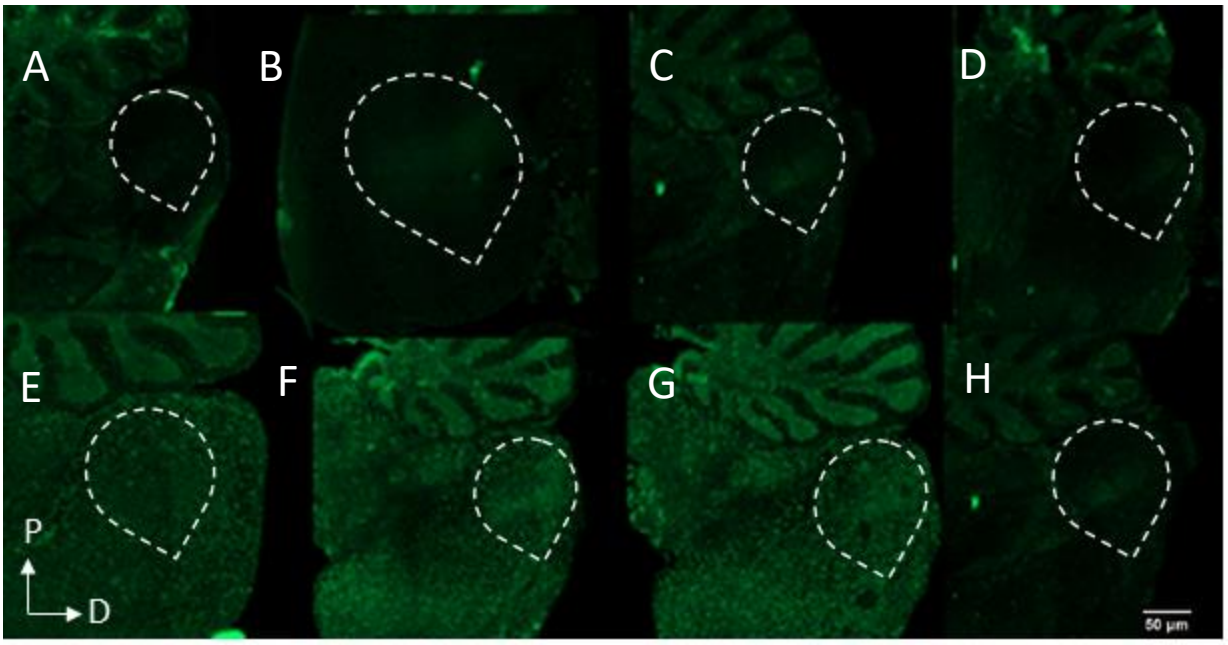

Figure S1 Comparison of the ISH results to the expected ISH results.
The ISH experiment consisted of all four miR probes with a hybridization temperature of 80˚C on adult male tissue. Panels A-D show the initial results – there was no signal produced from any of the four probes. Panels E-H show the expected results - signal produced from miR-124, miR-2954-5p, and miR-2954-3p, but not signal from the scramble probe. The expected results were generated after the protocol from Lin et al. 2014 was adjusted and optimized. Panel A and E show images of the miR-2954-5p probe. Panels B and F show images of the miR-2954-3p probe. Panels C and G show images of the miR-124 probe. Panels D and H show images of ISH using the scrambled miR probe. The auditory forebrain is outlined in white oriented according to the axis in the lower-left hand corner (P = posterior D = dorsal). A scale bar is included in the lower right-hand corner denoting 50µm. All images were taken with the 5X magnification lens.

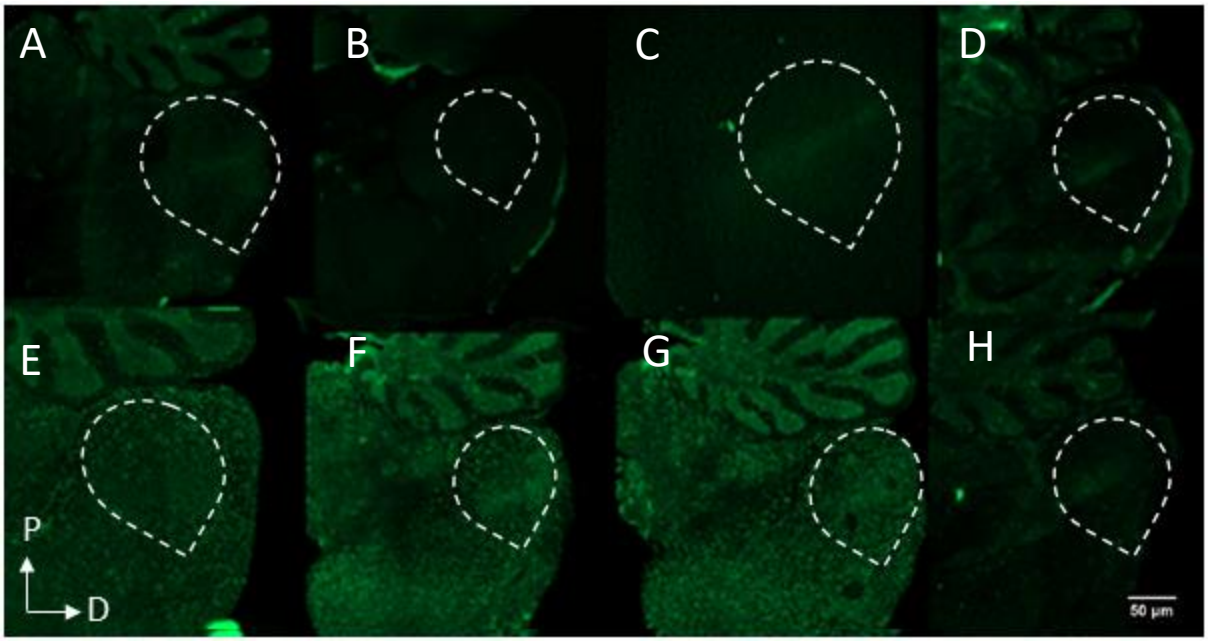

Figure S2 Comparison of the ISH results to the expected ISH results.
The ISH experiment consisted of all four miR probes with a hybridization temperature of 80˚C on adult male tissue after the tissue had been treated with the optimized Proteinase K solution. Panels A-D show the initial results – there was no signal produced from any of the four probes. Panels E-H show the expected results - signal produced from miR-124, miR-2954-5p, and miR-2954-3p, but not signal from the scramble probe. The expected results were generated after the protocol from Lin et al. 2014 was adjusted and optimized. Panels A and E show images of the miR-2954-5p probe. Panels B and F show images of the miR-2954-3p probe. Panels C and G show images of the miR-124 probe. Panels D and H show images of ISH using the scrambled miR probe. The auditory forebrain is outlined in white oriented according to the axis in the lower left-hand corner (P = posterior D = dorsal). A scale bar is included in the lower right-hand corner denoting 50µm. All images were taken with the 5X magnification lens.

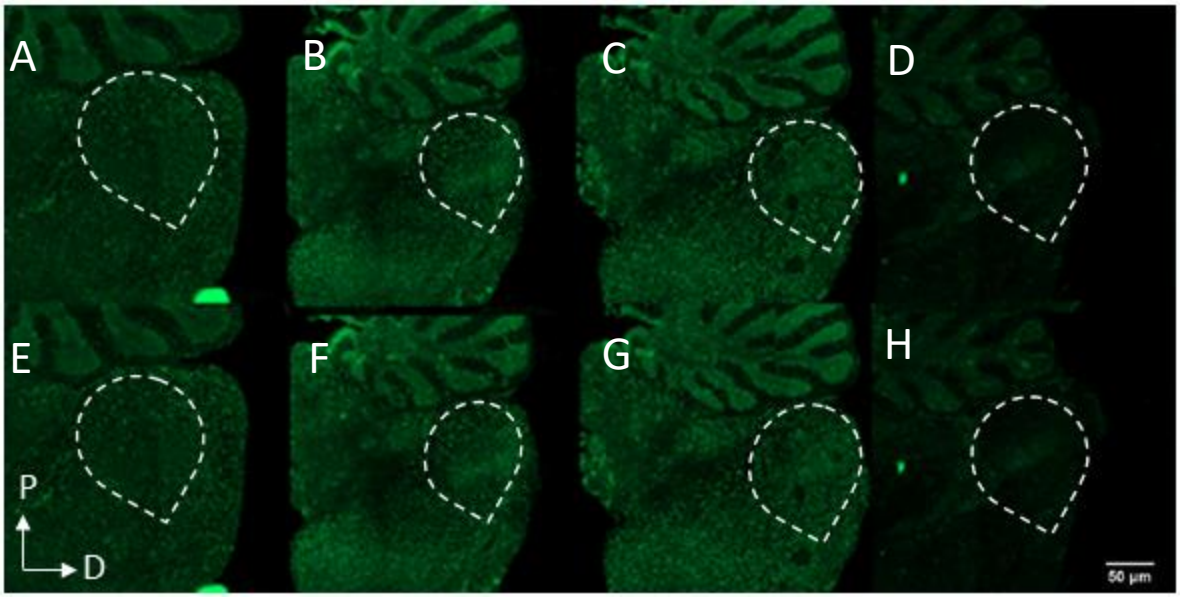

Figure S3 Comparison of the ISH results to the expected ISH results.
The ISH experiment consisted of all four miR probes with a hybridization temperature of 80˚C in a new hybridization oven on adult male tissue after the tissue had been treated with the optimized Proteinase K solution. Panels A-D show the initial results – there was no signal produced from any of the four probes. Panels E-H show the expected results - signal produced from miR-124, miR-2954-5p, and miR-2954-3p, but not signal from the scramble probe. The expected results were generated after the protocol from Lin et al. 2014 was adjusted and optimized. Panels A and E show images of the miR-2954-5p probe. Panels B and F show images of the miR-2954-3p probe. Panels C and G show images of the miR-124 probe. Panels D and H show images of ISH using the scrambled miR probe. The auditory forebrain is outlined in white oriented according to the axis in the lower left-hand corner (P = posterior D = dorsal). A scale bar is included in the lower right-hand corner denoting 50µm. All images were taken with the 5X magnification lens.

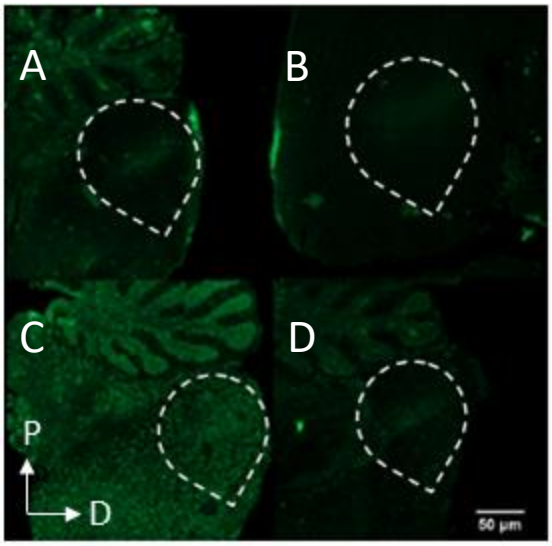

Figure S4 Comparison of the ISH results to the expected ISH results.
The ISH experiment consisted of the positive, miR-124, and negative, scrambled, miR probes with a hybridization temperature of 80˚C in the new hybridization oven on adult male tissue after the tissue had been treated with the optimized Proteinase K solution. Panels A and B show the initial results – there was no signal produced from either the positive or negative control. Panels C and D show the expected results - signal produced from the positive control, but no signal from the scramble probe. The expected results were generated after the protocol from Lin et al. 2014 was adjusted and optimized. Panels A and C show images of the miR-124 probe. Panels B and D show images of ISH using the scrambled miR probe. The auditory forebrain is outlined in white oriented according to the axis in the lower left-hand corner (P = posterior D = dorsal). A scale bar is included in the lower right-hand corner denoting 50µm. All images were taken with the 5X magnification lens.

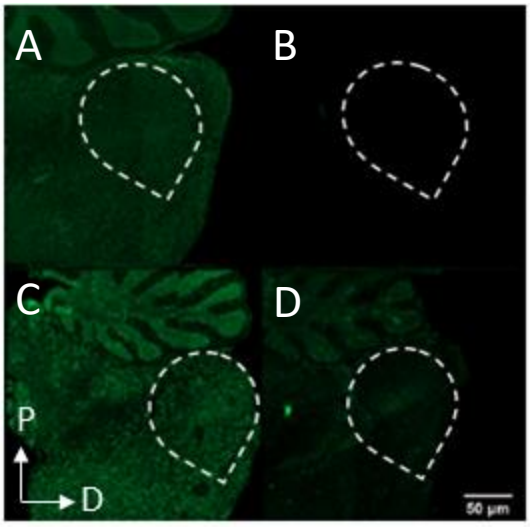

Figure S5 Comparison of the ISH results to the expected ISH results.
The ISH experiment consisted of the positive, miR-124, and negative, scrambled, miR probes with a hybridization temperature of 80˚C in the new hybridization oven after the racks had been adjusted on adult male tissue after the tissue had been treated with the optimized Proteinase K solution. Panels A and B show the initial results – there was signal from the positive control, but not the negative control. Panels C and D show the expected results - signal produced from the positive control, but no signal from the scramble probe. The expected results were generated after the protocol from Lin et al. 2014 was adjusted and optimized. Panels A and C show images of the miR-124 probe. Panels B and D show images of ISH using the scrambled miR probe. The auditory forebrain is outlined in white oriented according to the axis in the lower left-hand corner (P = posterior D = dorsal). A scale bar is included in the lower right-hand corner denoting 50µm. All images were taken with the 5X magnification lens.

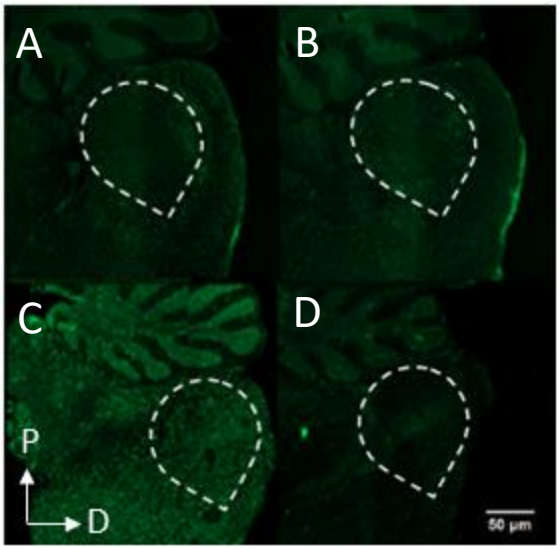

Figure S6 Comparison of the ISH results to the expected ISH results.
The ISH experiment consisted of the positive, miR-124, and negative, scrambled, miR probes with a hybridization temperature of 80˚C in the new purchased hybridization oven after the racks had been adjusted on adult male tissue after the tissue had been treated with the optimized Proteinase K solution. This tissue was placed on the top rack in the hybridization oven. Panels A and B show the initial results – there was signal produced from both the positive and negative control. Panels C and D show the expected results - signal produced from the positive control, but no signal from the scramble probe. The expected results were generated after the protocol from Lin et al. 2014 was adjusted and optimized. Panels A and C show images of the miR-124 probe. Panels B and D show images of ISH using the scrambled miR probe. The auditory forebrain is outlined in white oriented according to the axis in the lower left-hand corner (P = posterior D = dorsal). A scale bar is included in the lower right-hand corner denoting 50µm. All images were taken with the 5X magnification lens.

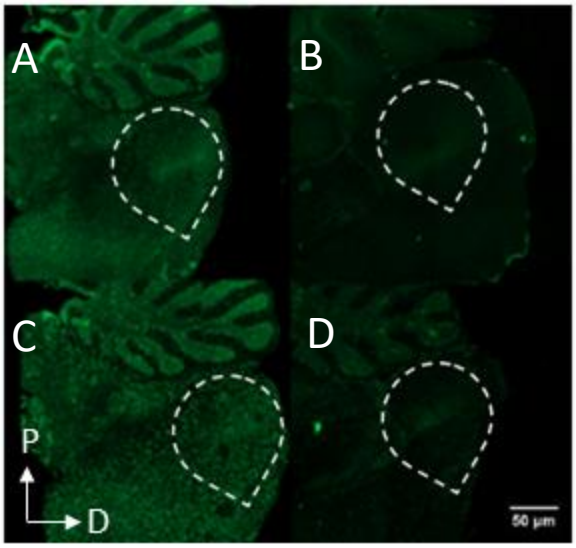

Figure S7 Comparison of the ISH results to the expected ISH results.
The ISH experiment consisted of the positive, miR-124, and negative, scrambled, miR probes with a hybridization temperature of 80˚C in the new hybridization oven after the racks had been adjusted on adult male tissue after the tissue had been treated with the optimized Proteinase K solution. This tissue was placed on the middle rack in the hybridization oven. Panels A and B show the initial results – there was signal produced from the positive, but not the negative control. Panels C and D show the expected results - signal produced from the positive control, but no signal from the scramble probe. The expected results were generated after the protocol from Lin et al. 2014 was adjusted and optimized. Panels A and C show images of the miR-124 probe. Panels B and D show images of ISH using the scrambled miR probe. The auditory forebrain is outlined in white oriented according to the axis in the lower left-hand corner (P = posterior D = dorsal). A scale bar is included in the lower right-hand corner denoting 50µm. All images were taken with the 5X magnification lens.

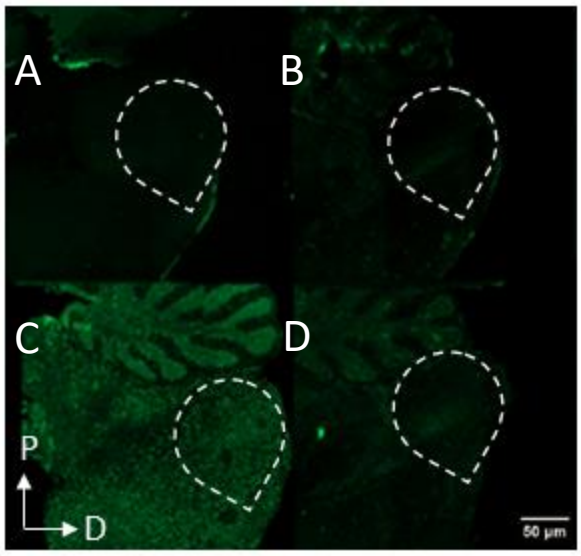

Figure S8 Comparison of the ISH results to the expected ISH results.
The ISH experiment consisted of the positive, miR-124, and negative, scrambled, miR probes with a hybridization temperature of 80˚C in the new hybridization oven after the racks had been adjusted on adult male tissue after the tissue had been treated with the optimized Proteinase K solution. This tissue was placed on the lowest rack in the hybridization oven. Panels A and B show the initial results – there was no signal produced from either the positive or negative control. Panels C and D show the expected results - signal produced from the positive control, but no signal from the scramble probe. The expected results were generated after the protocol from Lin et al. 2014 was adjusted and optimized. Panels A and C show images of the miR-124 probe. Panels B and D show images of ISH using the scrambled miR probe. The auditory forebrain is outlined in white oriented according to the axis in the lower left-hand corner (P = posterior D = dorsal). A scale bar is included in the lower right-hand corner denoting 50µm. All images were taken with the 5X magnification lens.

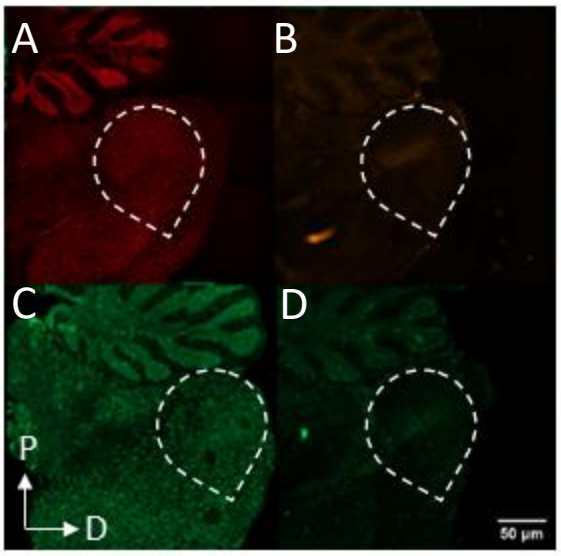

Figure S9 Comparison of the ISH results to the expected ISH results.
The ISH experiment consisted of the positive, miR-124, and negative, scrambled, miR probes with a hybridization temperature of 80˚C in the new hybridization oven after the racks had been adjusted on adult male tissue after the tissue had been treated with the optimized Proteinase K solution. This tissue was placed on the top rack in the hybridization oven. Panels A and B show the initial results – signal produced from the positive control, but no signal from the scramble probe. Panels C and D show the expected results - signal produced from the positive control, but no signal from the scramble probe. The expected results were generated after the protocol from Lin et al. 2014 was adjusted and optimized. Panels A and C show images of the miR-124 probe. Panels B and D show images of ISH using the scrambled miR probe. The auditory forebrain is outlined in white oriented according to the axis in the lower left-hand corner (P = posterior D = dorsal). A scale bar is included in the lower right-hand corner denoting 50µm. All images were taken with the 5X magnification lens.

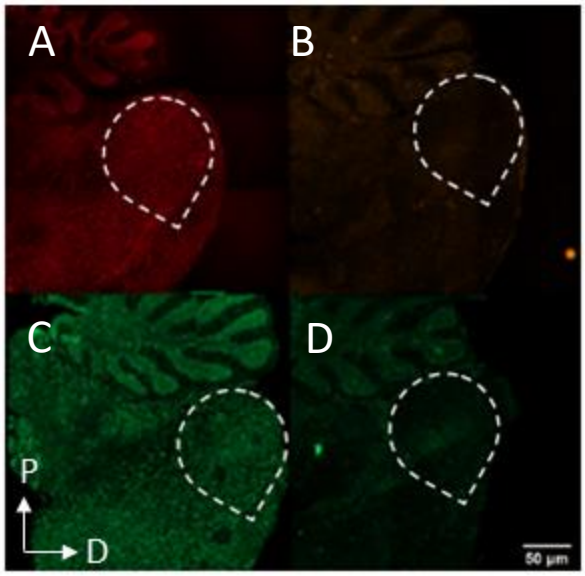

Figure S10 Comparison of the ISH results to the expected ISH results.
The ISH experiment consisted of the positive, miR-124, and negative, scrambled, miR probes with a hybridization temperature of 80˚C in the new hybridization oven after the racks had been adjusted on adult male tissue after the tissue had been treated with the optimized Proteinase K solution. This tissue was placed on the middle rack in the hybridization oven. Panels A and B show the initial results – signal produced from the positive control, but no signal from the scramble probe. Panels C and D show the expected results - signal produced from the positive control, but no signal from the scramble probe. The expected results were generated after the protocol from Lin et al. 2014 was adjusted and optimized. Panels A and C show images of the miR-124 probe. Panels B and D show images of ISH using the scrambled miR probe. The auditory forebrain is outlined in white oriented according to the axis in the lower left-hand corner (P = posterior D = dorsal). A scale bar is included in the lower right-hand corner denoting 50µm. All images were taken with the 5X magnification lens.

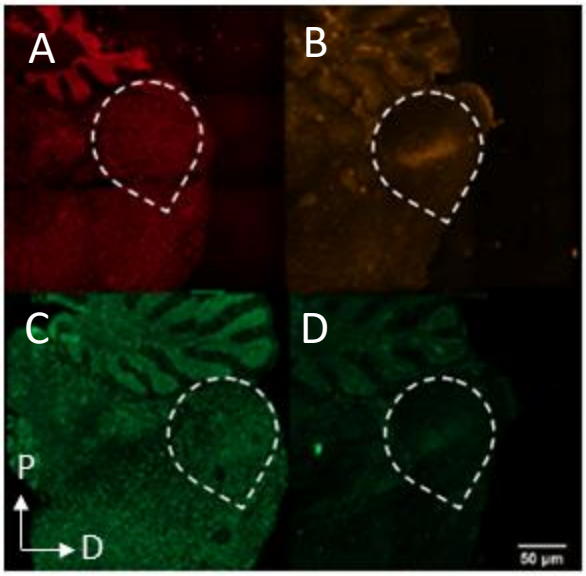

Figure S11 Comparison of the ISH results to the expected ISH results.
The ISH experiment consisted of the positive, miR-124, and negative, scrambled, miR probes with a hybridization temperature of 80˚C in the new hybridization oven after the racks had been adjusted on adult male tissue after the tissue had been treated with the optimized Proteinase K solution. This tissue was placed on the lowest rack in the hybridization oven. Panels A and B show the initial results – signal produced from the positive control, but no signal from the scramble probe. Panels C and D show the expected results - signal produced from the positive control, but no signal from the scramble probe. The expected results were generated after the protocol from Lin et al. 2014 was adjusted and optimized. Panels A and C show images of the miR-124 probe. Panels B and D show images of ISH using the scrambled miR probe. The auditory forebrain is outlined in white oriented according to the axis in the lower left-hand corner (P = posterior D = dorsal). A scale bar is included in the lower right-hand corner denoting 50µm. All images were taken with the 5X magnification lens.

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I was angry with my friend;
I told my wrath, my wrath did end.
I was angry with my foe:
I told it not, my wrath did grow.

And I watered it in fears,
Night & morning with my tears:
And I sunned it with smiles,
And with soft deceitful wiles.

And it grew both day and night.
Till it bore an apple bright.
And my foe beheld it shine,
And he knew that it was mine.

And into my garden stole,
When the night had veiled the pole;
In the morning glad I see;
My foe outstretched beneath the tree.