NEURAL RESPONSES TO AUDITORY RHYTHMS IN THE ZEBRA FINCH

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ABSTRACT

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Rhythm is important in the production of motor sequences such as speech and song. Deficits in rhythm processing have been implicated in a range of human disorders including some that affect speech and language processing, including stuttering, specific language impairment, and dyslexia. Songbirds provide a tractable model for studying the neural underpinnings of rhythm processing due to parallels with humans in neural structures and vocal learning patterns. In the experiments conducted for this dissertation, I investigated the effect of rhythmicity of song stimuli on expression of the immediate early gene ZENK in the adult zebra finch brain. I also investigated development of rhythmic discrimination in the juvenile brain, and estradiol (E2) effects on rhythm perception in adult birds.

In adult zebra finches, ZENK was increased in response to arrhythmic compared to rhythmic song in the auditory association cortex homologs, caudomedial nidopallium (NCM) and caudomedial mesopallium (CMM), and the avian amygdala, nucleus taeniae (Tn). CMM also had greater ZENK labeling in females than males. These auditory areas may be detecting errors in arrhythmic song when comparing it to a stored template of how conspecific song is expected to sound. CMM may also be important for females in evaluating songs of potential mates. Increased neural activity in Tn may be related to the value of song for assessing mate choice and bonding or to perception of arrhythmic song as aversive.

Before formation of the template for song that young birds memorize, expression of ZENK was increased in NCM of birds exposed to rhythmic relative to arrhythmic song. During template formation, ZENK expression was increased in CMM of birds exposed to arrhythmic relative to rhythmic song. These results suggest that the youngest birds may be predisposed to respond to a more natural stimulus, and a template may be required for arrhythmic song to elicit

increased neural activity. Rhythm discrimination in CMM may be strongest at life stages, such as during template memorization, when birds are most focused on external auditory signals. Compared to data from adults, it also appears that functional development across the brain regions investigated continues to maturity.

In adult zebra finches treated with a control or E2 or the aromatase inhibitor fadrozole (to increase or decrease estrogen availability), ZENK mRNA was significantly greater in the left hemisphere within NCM, CMM, and Tn. The overall pattern for left lateralization parallels the left lateralization of language processing in humans and may suggest that this hemisphere is specialized for processing conspecific vocalizations. Main effects of sex were detected in both auditory regions, with increased ZENK in males in NCM and in females in CMM. The reversed sex differences in NCM and CMM suggest that males and females differentially rely on components of the auditory forebrain for processing conspecific song. In CMM, an effect of hormone treatment also existed. While no pairwise comparison was statistically significant, the pattern suggested greater ZENK expression in control compared to both fadrozole- and E2-treated birds. In NCM, an interaction between sex and hormone treatment suggested that the sex effect was restricted to control animals. The hormone effects suggest that an optimal level of estradiol may exist for processing rhythmicity of auditory stimuli.

Together, these studies represent the first step in establishing the zebra finch as a model for human rhythm perception and disorders with disruptions in rhythm processing. This work suggests multiple brain regions that should be assessed in more detail for their involvement in human rhythm processing and disorders. A potential for a learned aspect of rhythm discrimination is also indicated, suggesting that rhythm training may aid those with disorders involving rhythm processing deficits. In addition, the establishment of the zebra finch as a model provides the opportunity to conduct more mechanistic studies into the basis of rhythm perception.

For David A. Lampen Sr., a brilliant mind. No one could ever believe in me like you did.

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KEY TO ABBREVIATIONS

Lateral magnocellular nucleus of the anterior nidopallium	LMAN
Robust nucleus of the arcopallium	RA
Caudomedial nidopallium	NCM
Caudomedial mesopallium	СММ
Nucleus taeniae	Tn
Medial nucleus of the dorsolateral thalamus	DLM
Tracheosyringeal part of the hypoglossal nucleus	nXIIts
Estradiol	E2
Phosphate buffered saline	PBS
15-17 days post hatching	d15
25-27 days post hatching	d25
45-47 days post hatching	d45
Maleic acid buffer with 0.1% tween-20	MABT

INTRODUCTION

Human Rhythm Perception

Rhythm is an important aspect of human speech, linguistic ability, and auditory processing. A positive correlation exists between rhythm perceptual abilities and language and literacy skill [1]. Many human disorders involve disruptions in rhythm perception and production. For example, people who stutter appear to have deficits in internal rhythm generation and timing for speech, but can produce fluent speech when provided with external rhythms by a metronome [2], another speaker [3], or singing [4]. Children with specific language impairment (language delay) have deficits in timing perception as well as in the ability to move in synchrony with a beat [5, 6]. It has been proposed that autism is based on deficits in temporal processing [7]; presentation of auditory rhythms may be able to alleviate some symptoms [8]. Disruptions in perception of timing have also been observed in patients with attention deficit hyperactivity disorder (reviewed in [9]), schizophrenia (reviewed in [9]), and dyslexia [10]. Parkinson's disease patients also show significant impairment in rhythm perception [11]. Thus, a better understanding of the neural basis of rhythm processing could elucidate mechanisms associated with a wide range of human speech, developmental and psychiatric disorders.

Stuttering is one of the disorders involving significant deficits in rhythm perception and production. Children who stutter have a reduced ability to discriminate complex rhythms as compared to their age-matched peers [12]. Stuttering is a communication disorder that is present in approximately 1% of all people [13]. The lifetime incidence of stuttering is approximately 5%, but estimates of spontaneous recovery are as high as 80% [13], indicating that many children who stutter no longer do so by adulthood. It is unknown why some spontaneously recover, and it presently is not possible to predict who will recover. Stuttering is highly sexually dimorphic in its prevalence, affecting approximately 3 times as many boys as girls, and this difference is larger in adulthood due to higher rates of spontaneous recovery in females [13]. People who stutter in adolescence show lower cognitive test scores [14], are

more likely to experience bullying [14, 15], have lower self-esteem [15], and report less optimistic life orientation [15] as compared to those who do not stutter. Given these negative outcomes, it is important to gain a deeper understanding of the neural mechanisms underlying stuttering in order to eventually develop new therapeutic approaches.

In human children, rhythmic perceptual abilities are present early in life, but they continue to mature through development. Infants appear to be attuned to natural speech rhythms at 9 months, but not at 6 months [16]. As early as 2 months of age, infants are capable of discriminating between rhythmic patterns [17]. While perceptual rhythm abilities develop in the first few months of life, ability to entrain to a beat is not present until 4 years of age (reviewed in [18]). Perception and discrimination of tempi appears to improve with age in children, adolescents and young adults [19-21]. Children typically perceive faster tempi more easily than slower tempi [19]. Rhy1thmic processing appears to influence language and literacy skills. For instance, a correlation exists between literacy and brainstem responses to auditory rhythm in children [22]. Auditory rhythm processing ability is positively correlated with and literacy and linguistic skill in children [23]. In addition, in children struggling with reading, a rhythm based intervention that did not involve any specific linguistic training was equally effective at improving reading skills as an established phoneme-grapheme training intervention [24], indicating the importance of rhythm processing for literacy and that rhythm deficits alone may significantly impair reading. Together these data demonstrate the importance of children's developing rhythmic capacity for language and literacy learning.

The perception and processing of rhythm is a complex process that involves many regions of the brain. In adult humans, greater activation in the supplementary motor area of the cortex and the basal ganglia is detected in response to regular rhythms compared to complex, irregular rhythms [25]. Additional support for the role of the basal ganglia in rhythm perception comes from Parkinson's disease patients who experience degeneration of the basal ganglia and lack the perceptual advantage provided by beat based compared to complex rhythms [11]

typically seen in healthy participants [25]. In addition, stutterers show less activation of the basal ganglia during a speech task as compared to non-stutterers [26], providing support to the idea that stutterers may have impaired rhythmic processing. Other areas involved in rhythm perception include the premotor [27] and auditory cortex [28]. An understanding of how these brain areas function together in rhythm perception and how they may be deficient in people who stutter, as well as people with other disorders involving disrupted rhythm perception, is still lacking.

Zebra Finches as a Model to Investigate Rhythm Perception

Zebra finches represent a tractable model for studying neural mechanisms of timing and rhythm perception and their relationship to vocalization. Both humans and zebra finches are vocal learning species and go through similar stages of memorizing adult vocalizations, practicing vocal output in an effort to match the memorized template, and ultimately producing their own adult vocalizations (reviewed in [29]). Vocal learning has been suggested as a necessary factor for the capacity to perceive and entrain to rhythmic auditory stimuli [30]. However, recent evidence suggests it may aid in rhythmic perception, but not be required; the sea lion, which is not a vocal learner, has demonstrated the capacity to entrain to a beat [31, 32]. The extent to which vocal learning species than species that do not learn their vocalizations have demonstrated a capacity to entrain to a beat [34]. Zebra finches produce highly rhythmic vocalizations [35] used for courtship and the defense of nest sites [36]. This consistent natural rhythm of zebra finch song [35, 36] provides a unique opportunity to study the neural basis of rhythm perception and its development in a vocal learner.

Rhythm Perception in Birds

Recent research has added greatly to the understanding of the rhythmic abilities of birds, and particularly zebra finches. Analysis of natural zebra finch song demonstrates that it contains a natural pulse that typically coincides with note onsets and neuromuscular gesture

transitions within notes, suggesting that songs are organized in a non-random way to maintain an underlying temporal structure [35]. When zebra finches raised in isolation were used to start a breeding colony, the rhythmic structure of the songs became increasingly similar to wild-type song with subsequent generations [37], suggesting that there may be some innate tendency toward a regular time structure in zebra finch song. The timing of zebra finch calls is also quite regular, with birds responding to calls with a stereotyped latency. When a robot was set to call during the expected time of the responding bird, both male and female zebra finches were able to quickly adapt their calls to avoid calling simultaneously with the robot [38], indicating the ability to judge local time intervals.

Whether zebra finches and other birds are capable of perceiving higher order rhythms or are simply focused on local timing elements is an issue still under debate [33, 39]. In a go/no go paradigm where birds were asked to discriminate between regular and irregular beat patterns zebra finches seemed to default to making decisions based on local temporal structure, such as duration of notes or inter-onset intervals, but also appeared capable of detecting broader temporal structure, encompassing multiple shorter intervals [33]. A similar study judged birds' capacity to discriminate between isochronous and irregular tone sequences [40]. While zebra finches could discriminate between the training stimuli, they were not able to generalize to isochronous rhythms at different tempi, which was interpreted to mean that they were only able to attend to local timing features [40]. A preferred tempo range exists in humans outside of which individuals are unable to accurately entrain to a beat [41]. If the same type of limitation of tempo perception is present in birds, it is possible that the limitations in zebra finches' abilities to generalize rhythm discrimination across tempi may not be due to an inability to perceive global rhythm, but instead the changed tempi may be outside of their perceptual range, thus decreasing their capacity to discriminate rhythmic stimuli. In addition, regions of the zebra finch brain do not respond as strongly to tones as conspecific vocalizations [42-46], thus the capacity to process global rhythms may be different if the sound pattern is composed of natural zebra

finch sounds which induce more activity in auditory processing areas of the brain. The overall capacity of zebra finches to attend to different levels of timing and rhythm requires further investigation.

Song Development

Rhythm perception may be a learned characteristic in zebra finches, acquired during the development of an adult song template. The general timeline of vocal development in zebra finches is agreed upon, but the exact ages at which specific milestones occur is still somewhat debated [47-50]. While only males sing, template formation likely occurs in both sexes approximately between post-hatching days 20 and 60 [48-50]. Females likely acquire a song template from their fathers [51], which is presumably used as a model for quality in the selection of potential mates [36, 52]. In males, vocalizations begin as food begging, then develop into subsong, which is an immature form of song that is quiet and contains poorly formed notes with greatly variable structure and sequence [36]. As the males mature, these songs are practiced and modified to relatively closely match the learned template. This phase of sensorimotor integration occurs between about post hatching day 25 and sexual maturity at around 90 days of age [48-50]. After sensorimotor integration males have a crystalized song that will not undergo any significant changes throughout their lives under normal circumstances [47].

Neural Song Circuit

Humans and zebra finches have substantial parallels in the neural structures underlying the perception, learning, and production of vocalizations [53]. Area X, part of an anterior circuit that is involved in song learning, is not visible in female zebra finches [54, 55] who do not sing, and is homologous to the human striatum [56, 57] which is involved in language learning [58] (Figure 1). The lateral magnocellular nucleus of the anterior nidopallium (LMAN) is part of the circuit essential for song learning, and is necessary for song plasticity during development [59-61] and in adulthood [62-64]. A homologous region to LMAN within the human brain has not been identified, but it is described as a frontal cortical nucleus [65]. HVC (proper name) is

similar to the premotor cortex in humans [65], and is involved in timing in song production [66]. The robust nucleus of the arcopallium (RA) is similar to the motor cortex in humans [65, 67] and is responsible for generating the spectral features of song [66]. Both of these areas are larger in male than in female zebra finches [54, 55]. The caudomedial nidopallium (NCM) and caudomedial mesopallium (CMM), while anatomically distinct in the zebra finch brain, are both considered homologous to the auditory association cortex in humans [68].

The anterior forebrain pathway is composed of Area X, LMAN, the medial nucleus of the dorsolateral thalamus (DLM), and their

projections. Area X receives inputs from LMAN and HVC and sends output to DLM. LMAN receives input from DLM and sends output to Area X and RA. The vocal motor pathway is composed of HVC, RA, and the tracheosyringeal part of the hypoglossal nucleus (nXIIts). HVC receives input from auditory areas including NCM, CMM, and field L, the avian primary auditory cortex homolog [69], and sends output to Area X and RA.



RA receives input from HVC and LMAN and projects to nXIIts which innervates the vocal organ (syrinx). The cortical auditory structures in the bird brain include field L, NCM, and CMM. Field L sends output to NCM and CMM. Reciprocal connections are present between NCM and CMM, and both nuclei project to HVC (Figure 1).

<u>ZENK</u>

Many studies have investigated zebra finch auditory perception and the factors influencing neural responses to auditory stimuli. A marker commonly used to assess neural activation in zebra finches is the immediate early gene ZENK [43, 45, 70-76]. As an immediate early gene ZENK is expressed in neuron following depolarization and can be used as a marker of neural activity. 'ZENK' is an acronym used to identify the evolutionally conserved protein based on names from other species, specifically zif-268 [77], egr-1 [78], NGFI-A [79], and Krox-24 [80]. The ZENK protein has a DNA binding site and can regulate the expression of other genes [81]. It is involved in synaptic plasticity, and memory [82]. If the pathway for ZENK induction is interrupted in juvenile zebra finches, song learning is significantly reduced [83].

Both male and female zebra finches show robust induction of ZENK in multiple brain regions in response to conspecific song [70]. For example, NCM and CMM express high levels of ZENK following zebra finch song playbacks [43, 74, 75]. Investigations have also repeatedly demonstrated increased ZENK with presentation of conspecific compared to heterospecific song, pure tones, or silence in both adults [43, 44] and juveniles [45, 70]. The increase in neural activity in response to conspecific song has been demonstrated using other methodologies such as fMRI [84], and electrophysiology [85]. ZENK expression in NCM and CMM is also greater in response to song from tutored compared to untutored zebra finch males [76, 86]. Immediate early gene expression following tutor song presentation is significantly correlated with the strength of song learning [87, 88]. Together these results suggest that ZENK expression in auditory cortical regions is highest in response to stimuli that are most similar to the song template learned during development.

Distinct patterns of ZENK expression suggest that specific brain regions are activated by *hearing* song and others are associated with *producing* it [73, 75]. In canaries, singing induces ZENK expression in HVC, RA, LMAN, and Area X among other nuclei [73]. Hearing conspecific song creates a different pattern in canaries, with abundant expression in portions of the primary auditory cortex homolog field L, as well as CMM and NCM [73]. Parallel patterns of ZENK expression are seen in zebra finches that sing or hear conspecific song [75]. In zebra finches, HVC also shows electrophysiological responses to a bird's own song [89] and tutor's song [90],

with much less response to other conspecific songs. However, no difference in ZENK induction was detected between birds exposed to their own song, tutor song, or novel conspecific song [71]. Analyses of ZENK expression do not always show the same activity patterns as electrophysiological measurement of neural responses; it is possible for neurons to fire without inducing ZENK expression (reviewed in [91]).

ZENK in Development

While the expression of ZENK in the adult zebra finch brain has been characterized in a variety of contexts, the development of ZENK responses to auditory stimuli has been less well studied. At post-hatching day 20, there is high constitutive expression of ZENK in NCM, and ZENK induction by song is possible at day 30 [42, 92]. In 30 and 45 day old zebra finches conspecific song, but not heterospecific song or tones, induces ZENK expression uniformly throughout the medial and lateral portions of the medial striatum in females and within the medial striatum, but not in Area X, which is located in the lateral portion of the medial striatum in males [46]. ZENK expression in the NCM, CMM, and hippocampus is greater in response to conspecific song than heterospecific song, tones, or silence in male, but not female, zebra finches at post-hatching day 30 [45]. At day 45, normal zebra finch song induces greater ZENK expression than untutored song in NCM and CMM of both sexes, but these effects are not seen in birds that were raised without a tutor [76], indicating that a learned template is necessary for normal song discrimination. In day 45 zebra finches of both sexes, conspecific song induces greater ZENK expression in NCM than silence, but differences are not seen between conspecific and heterospecific song or in CMM among the stimuli [70]. Further research is needed to clarify the developmental patterns of song discrimination various auditory responsive nuclei.

Estradiol and Perception

Estradiol (E2) can broadly influence perceptual systems in a large range of animals. Estrogen receptors are present in many sensory organs. For example, ERα and ERβ have

been found in the human olfactory neuroepithelium [93]. Estrogen receptors also exist in rat dorsal root ganglion cells, and multiple tissues in the eye, including the retina, in rats, rabbits, humans, cows, and trout [94-96]. Cochlear estrogen receptors have also been described in mice, rats, zebra finches, and an African cichlid fish [97-99]. The abundance of estrogen receptor in sensory organs provides great opportunity for hormone modulation of perception in various modalities. E2 improves olfactory perception in women with postmenopausal hormone therapy [100] and in female rats [101]. In the peak fertile phase of the menstrual cycle, which is when E2 levels are highest, women in relationships have a preference for odors of more dominant men, not seen during other times of the cycle [102]. Women with low levels of E2 show attenuation of auditory event-related potentials in the cortex [103]. Auditory brainstem responses are also influenced by E2 levels, with E2 replacement decreasing latencies in ovariectomized rats [104]. Decreased latencies are also detected in E2-treated juvenile female rhesus monkeys [105], as well as women in the periovular phase of the menstrual cycle, when E2 levels are increased [106].

Estradiol's Effect on Responses to Song

A variety of techniques have been utilized to assess the impact of E2 on responses to song, including local infusion of E2 in particular brain regions, systemic E2 administration, electrophysiology, ZENK assessment, and analysis of behavioral responses. Electrophysiological data has shown some inconsistency in how E2 infused into NCM influences selectivity of local neurons. For example, E2 consistently increases responses to conspecific song and a bird's own song, but the data are inconsistent on whether it increases responses to reverse song, heterospecific song, or white noise [107-109]. The effects of fadrozole in NCM on responses to auditory stimuli is also inconsistent, with reports of reduced activity [108] or no effect [109].

The effects of E2 on ZENK expression in the brain have been well studied in whitethroated sparrows. These birds breed seasonally and experience regression of the gonads

during the nonbreeding season, leading to very low circulating E2 levels [110]. In both NCM and CMM, no differences are seen in ZENK expression in the non-breeding season following exposure to conspecific song or tones, but with systemic E2 replacement higher ZENK is seen in song exposed birds [110]. These effects are not due to an increase in ZENK in response to conspecific song, but instead to a decrease in response to tones [110]. This result may suggest that E2 is important for selectivity of neural responses in these auditory areas, allowing for inhibition of responses to ecologically irrelevant stimuli. Similar patterns of ZENK expression are seen in brain regions within the social behavior network [111] including Tn, where birds in the breeding season have greater ZENK expression in response to song than to tones or silence, and E2 is able to induce this difference in non-breeding birds [111]. This suggest that E2 can also influence selectivity of neural responses in Tn, potentially through both increased responses to song and inhibition of responses irrelevant stimuli.

The influence of E2 on behavioral preferences for song types has been assessed in multiple ways. As measures of preference for different song types in female zebra finches, Vyas et al. (2009) quantified body shakes, orienting toward the speaker where song was being played, and long calls [112]. In birds implanted with E2, significant increases were detected in all three measures for complex song (containing a large number of unique syllables and variety in the syllables present in each motif) compared to long-bout songs (consisting of a large number of uniform motif repetitions resulting in bouts of long duration) or prototypical songs (shorter with simple repeated motifs); these differences in response to song type were not seen in a pretest without hormone treatment [112, 113]. The results following treatment with an aromatase inhibitor were more complex, with no differences seen in shakes, but an increase in orienting toward the speaker with complex song compared to long-bout song types [112]. These results suggest that E2 may differentially regulate various aspects of behavioral responses to conspecific vocalizations. Another test used to assess E2's influence on song preference

involves playing two different songs simultaneously at opposite ends of a long cage and assessing time spent near each speaker. Retrodialysis of the aromatase inhibitor fadrozole into NCM in the left, but not the right, hemisphere eliminated the increase in time spent near a speaker playing bird's own song compared to time near a speaker playing conspecific song in adult male zebra finches [109]. This suggests that E2 is necessary for this behavioral difference. It is unclear from these studies whether the differences in behavioral responses were due to an influence of E2 on the neural processing of auditory stimuli or their salience. <u>Estradiol in the Zebra Finch Auditory and Song Systems</u>

The presence of both aromatase, the enzyme responsible for the production of E2 by metabolism from testosterone, and estrogen receptors within auditory responsive nuclei in the brain provides the opportunity for E2 to influence auditory processing. Aromatase expression is abundant in the zebra finch brain, with similar patterns detected for mRNA [114] and protein [115, 116]. The enzyme is highly expressed within nucleus taeniae (Tn), the avian homolog of the mammalian amygdala, as well as NCM and CMM, but appears absent from song system nuclei LMAN, Area X, HVC, and RA [114-116]. Areas that produce E2 also have the capacity to respond to the hormone. For example, ERα mRNA [117] and protein [118] are present in both NCM and Tn. GPR30 is a membrane bound estrogen receptor that is involved in rapid responses to E2 [119]. The expression of GPR30 has been mapped in the juvenile and adult zebra finch brain [120]. At post hatching day 21 dense GPR30 labeling is detected in Tn, HVC, and RA. Slightly less dense staining is seen in NCM and CMM. By adulthood GPR30 expression is largely reduced, but moderate labeling remains in areas with dense labeling during development [120].

Given the abundant expression of aromatase within NCM [114-116], it is not surprising that there is a high degree of local E2 synthesis within the auditory nucleus [121]. Interestingly, it occurs specifically in response to song. Microdialysis in NCM of awake, behaving zebra finches reveals a significant increase in E2 concentration during exposure to conspecific song in

both males [122] and females [108]. In male zebra finches, increased E2 was detected in NCM during exposure to silent video of a female zebra finch, but not during video of male zebra finches or Bengalese finches [108]. No increase in E2 was seen in response to any of the video types in the female NCM [108]. E2 increases in NCM were also observed in males when a female was placed adjacent to the testing cage [122]. These effects of song and social stimulus were specific to NCM because microdialysis just outside NCM or specifically in CMM did not show E2 responses to visual or auditory stimuli [108, 122]. These local increases in E2 in response to song may facilitate discrimination of auditory stimuli and promote maximal neural activity to the most ecologically relevant stimuli.

Summary of Dissertation Experiments

The first experiment in this dissertation was designed to test the hypothesis that the zebra finch brain is capable of discriminating between rhythmic and arrhythmic auditory stimuli, as indicated by expression of the immediate early gene ZENK. The next experiment further probed the basis of rhythmic processing ability by assessing neural responses to rhythmicity at multiple stages during vocal-motor development in order to test the hypothesis that a learned song template was necessary for differential neural responses to rhythmicity. With the final experiment in this dissertation, I sought to assess the hypothesis that rhythm processing in NCM, CMM and Tn is modulated by E2.

Chapter 1: ZENK expression in response to rhythmic and arrhythmic conspecific song in adult zebra finches. The density of cells expressing ZENK protein was analyzed within the anterior forebrain pathway, the vocal-motor song circuit, the auditory forebrain, and one region of the social behavior network (Tn) [123].

Chapter 2: Development of ZENK responses to rhythmic and arrhythmic song. Immunohistochemistry was used to assess density of ZENK+ cells across multiple stages of vocal learning in regions that showed significant effects of stimulus type in adults: NCM, CMM and Tn [124].

Chapter 3: E2 influences on ZENK responses to rhythmicity of auditory stimuli in adults. *In situ* hybridization was used to quantify the density of cells expressing ZENK mRNA in birds treated with E2, fadrozole, or a control manipulation (submitted manuscript).

CHAPTER 1

Arrhythmic Song Exposure Increases ZENK Expression in Auditory Cortical Areas and Nucleus Taeniae of the Adult Zebra Finch

Introduction

Human speech and avian song have many parallels: both are acquired through sensorimotor learning, and when well-formed they are rhythmically structured in time. There is increasing evidence that rhythm plays an important role in speech and language processing. During development, rhythm perception ability is positively correlated with language and literacy skill [1]. Moreover, children with specific language impairment (language delay) have deficits in rhythm processing that include the ability to move in synchrony with a beat [5, 6]. People who stutter also appear to have deficits in internal rhythm generation and timing for speech, but can produce fluent speech when paced by an external rhythm such as a metronome [2], another speaker [3], or singing [4]. A number of other human disorders also involve disruptions in timing and/or rhythm processing. For example, individuals with autism have been proposed to show deficits in temporal processing [7], with presentation of auditory rhythms possibly alleviating some symptoms [8]. Disruptions in aspects of timing or rhythm processing have also been observed in patients with attention deficit hyperactivity disorder (reviewed in [9]), schizophrenia (reviewed in [9]), and dyslexia [10]. Parkinson's disease patients also show significant impairment in rhythm perception [11]. Thus, a better understanding of the neural bases of rhythm processing could elucidate mechanisms associated with a wide range of human developmental and psychiatric disorders.

Zebra finches represent an excellent potential model for studying neural mechanisms of timing and rhythm perception. As songbirds, they produce highly rhythmic vocalizations used for courtship and the defense of nest sites [36]. Zebra finch song begins with a series of short introductory notes, followed by several repetitions of an ordered set of notes called a motif (Figure 2). A complete sequence of introductory notes and subsequent motifs performed without

a prolonged silent interval is referred to as one song bout. The intervals between notes are very regular. This consistent natural rhythm of zebra finch song [36] provides a relatively unique opportunity to study rhythm as a discriminatory characteristic. Furthermore, as an animal model, zebra finches provide an opportunity to study the neural basis of rhythm perception in a more direct manner than possible with humans.

Figure 2. Representative spectrograms of rhythmic and arrhythmic song. Images depict 7.5 seconds of representative rhythmic and arrhythmic song stimuli. They were generated from the same natural song. Introductory notes are indicated with I. A, B, and C indicate 3 distinct notes that compose a motif. Each image contains two bouts of song.



Zebra finches are vocal learners [29] and, similarly, normal human rhythm processing has been proposed to be a by-product of vocal learning mechanisms [30]. Moreover, as vocal learners, zebra finches learn to sing in a manner similar to the way humans develop speech (reviewed in [29]). Both species initially form an auditory template by listening to vocal production of adult tutors. They then practice and improve on their own vocalizations, which include subsong in birds and babbling in humans, and ultimately produce adult crystalized song in zebra finches and fluent speech in humans. In both species, critical periods exist after which vocal learning is strongly limited. In addition to the similar developmental trajectories, humans and zebra finches have substantial parallels in the neural structures underlying the perception, learning, and production of vocalizations [53]. Area X, part of an anterior circuit involved in song learning, is not visible in female zebra finches [54, 55] who do not sing, and is homologous to the human striatum [56, 57] which is involved in language learning [58]. HVC (proper name) is similar to the premotor cortex [65] and the robust nucleus of the arcopallium (RA) is similar to the motor cortex in humans [67]. Both HVC and RA are part of a motor circuit involved in song production. Both of these areas are larger in male than in female zebra finches [54, 55]. Although only male zebra finches sing, females likely also acquire a song template from their fathers [51, 125, 126], which is presumably used as a model for quality in the selection of potential mates [36, 52, 127]. The caudomedial nidopallium (NCM) and caudomedial mesopallium (CMM), while anatomically distinct in the zebra finch brain, are both considered homologous to the auditory association cortex in humans [68]. The lateral magnocellular nucleus of the anterior nidopallum (LMAN) is part of the circuit essential for song learning, and is necessary for song plasticity during development [59-61] and in adulthood [62-64]. A homologous region to LMAN within the human brain has not been identified.

Many studies have investigated zebra finch auditory perception and the factors influencing neural responses to auditory stimuli. A marker commonly used to assess neural activation in zebra finches is the immediate early gene ZENK [43, 45, 70-76]. 'ZENK' is an acronym used to identify the evolutionally conserved protein based on names from other species, specifically zif-268 [77], egr-1 [78], NGFI-A [79], and Krox-24 [80]. The ZENK protein has a DNA binding site and can regulate the expression of other genes [81]. It is thought to be involved in synaptic plasticity, and memory [82]. Extracellular signal-related kinase is an upstream component of the signaling pathway including ZENK; its inhibition blocks induction of ZENK in the zebra finch auditory cortex [72]. If the pathway for ZENK induction is interrupted in juvenile zebra finches, song learning is significantly reduced [83].

Both male and female zebra finches show robust induction of ZENK in multiple brain regions in response to conspecific song [70]. For example, NCM and CMM express high levels of ZENK following zebra finch song playbacks [43, 74, 75]. Investigations have also repeatedly demonstrated increased ZENK with presentation of conspecific compared to heterospecific song, pure tones, or silence in both adults [43, 44] and juveniles [45, 70]. The increase in neural activity in response to conspecific song has been demonstrated using other methodologies such as fMRI [84, 128], and electrophysiology [85]. ZENK expression in NCM and CMM is also greater in response to song from tutored compared to untutored zebra finch males [76, 86]. Immediate early gene expression following tutor song presentation is significantly correlated with the strength of song learning [87, 88]. Together these results suggest that ZENK expression in auditory cortical regions is highest in response to stimuli that are most similar to the song template learned during development.

Distinct patterns of ZENK expression suggest that specific brain regions are activated by *hearing* song and others are associated with *producing* it [73, 75]. In canaries, singing induces ZENK expression in HVC, RA, LMAN, and Area X, among other nuclei [73]. Hearing conspecific song creates a different pattern of ZENK expression in canaries, with abundant expression in portions of the primary auditory cortex homolog field L, as well as CMM and NCM [73]. Parallel patterns of ZENK expression are seen in zebra finches that sing or hear conspecific song [75]. In zebra finches, HVC also shows significant electrophysiological responses to a bird's own song in anesthetized subadults [89] and sleeping juveniles, and tutor's song in awake juveniles [90], with much less response to other conspecific songs. However, the same pattern has not been described in ZENK responses [71] when assessed in awake adults.

While many aspects of zebra finch auditory responses have been studied, little is known about their perception of rhythm, and whether rhythm is a salient factor in their discrimination of songs. To evaluate whether rhythmicity influences neural responses, and which brain regions

are involved in processing information about rhythmic structure in zebra finches, the present study exposed adult males and females to conspecific songs with normal structure ('rhythmic') or the same vocalizations with varied timing of syllable onset while maintaining the same syllable order ('arrhythmic'). The density of ZENK immunoreactive cells was assessed in several brain areas of interest, including regions important to song production and perception. Expression was also quantified in nucleus taeniae (Tn; a homolog to the mammalian amygdala, [129]), because an initial qualitative assessment indicated particularly strong labeling there. We hypothesized that ZENK expression would differ in birds exposed to rhythmic or arrhythmic song in nuclei involved in the perception or evaluation of auditory stimuli.

Materials and Methods

Animals

Zebra finches were raised in walk-in aviaries at Michigan State University, each containing 5-7 pairs of males and females with their offspring. Birds were maintained on a 12:12 light:dark cycle with lights turning on at 7am and provided *ad libitum* access to seed (Kaytee Finch Feed, Chilton, WI), water, gravel and cuttlebone. Their diet was supplemented weekly with hard boiled chicken eggs mixed with bread, as well as spinach and oranges. Once birds reached adulthood (at least 90 days of age), they were transferred to adjacent single sex walk-in aviaries and allowed a minimum of 10 days to acclimate to their new housing prior to experimental stimulus exposure. Birds could see and hear birds of the opposite sex, but could not physically interact. Animals in the study were less than 1 year old.

Ethics Statement

All protocols were approved by the Institutional Animal Care and Use Committee of Michigan State University (#01-13-006-00).

Stimulus creation

Nine 30-second rhythmic song stimuli and nine 30-second arrhythmic song stimuli were formed using Praat software [130] (Figure 2). To create these stimuli, nine zebra finch song recordings were selected from Boston University's Laboratory of Neural Circuit Information Zebra Finch song data set (http://people.bu.edu/timothyg/song website/index.html). For each stimulus, introductory syllables and two subsequent motifs (motif 1 and motif 2) were extracted from a recording. They were alternated 5 times, forming an alternating (1-2-1-2-1) motif structure. Thus a single bout of a song consisted of an unmanipulated sequence of introductory notes, followed by five unmanipulated motif productions (Figure 2). To form a complete rhythmic song stimulus, bouts were repeated for 30 seconds, with at least 0.4 seconds of silence between each bout presentation. The remaining silence, after repeating bouts until a complete bout could not be repeated without surpassing 30 seconds, was distributed evenly across the intervals between bouts so that each complete stimulus was 30 seconds. Across the rhythmic stimuli, silence between bouts ranged from 0.4 to 1.4 seconds (mean silence between bouts = 0.8 seconds). The 9 rhythmic song stimuli were divided into 3 groups of 3 such that the total length of silence was similar across groups. Maintaining a similar amount of total auditory stimulus across groupings was important because duration of song exposure can influence levels of ZENK expression [74].

To create the nine arrhythmic song stimuli, the length of each interval between syllables (other than between introductory notes), motifs, and bouts of the rhythmic song stimuli was altered using Matlab (The Mathworks, Inc., Natick, MA). The same total amount of spacing within the 30-second stimulus was retained. However, each interval was randomly changed to one of three durations: 1) 10 ms, 2) the average duration (based on all intervals in a song except those between introductory notes), or 3) double the average duration, minus 10 ms. After all but the final interval had been changed to one of those three durations, the final one in each song was changed to the duration needed to add up to the original total (Figure 2). In this manner, the

sequential order of syllables was preserved, but the rhythmicity, or regularity, of the timing of the syllables was disrupted, yielding arrhythmic songs. The 9 arrhythmic song stimuli were divided into 3 groups of 3 corresponding to the same grouping as the rhythmic stimuli. There were a total of 6 groups, 3 in the rhythmic condition, and 3 in the arrhythmic condition.

Song Exposure

For each stimulus type, 9 males and 9 females were exposed. Presentation of stimuli was controlled using E-Prime 2.0 software (Psychology Software Tools, Inc., Pittsburgh, PA). Individual birds were exposed to a stimulus inside an 11.25"x8.5"x15.25" cage within a 7'2"x14'9" room with lights on. First, a 1-hour period of silence allowed birds to acclimate to the testing room. Following the hour of silence, each bird was exposed to one group of 3 songs (either rhythmic or arrhythmic, randomly selected). Songs were played from a single speaker adjacent to the testing cage. Song stimuli were presented in pseudo-random order for a total of 30 presentations (10 presentations of each 30-second song), yielding a total of approximately 15 minutes of song. For every 3 presentations, each song was heard once. There were 30 seconds of silence between each song. Therefore, the song presentation portion of the procedure lasted approximately 30 minutes. All songs were played within the volume range of normal zebra finch song, at approximately 70 dB. Both the testing room and all stimuli were novel for all birds. All testing occurred between 9am and 3pm, with a maximum of two birds tested in a day. Different stimulus groups were randomized across morning and afternoon testing times. Following song exposure animals remained in the testing room for 1 hour undisturbed in order to allow ZENK protein expression to reach peak levels [75]. They were then euthanized by rapid decapitation, whole brains were collected and frozen in methylbutane. Brains were stored at -80°C until further processing.

All of the song exposures were recorded using a Canon Vixia HF R300 camcorder. Recordings of all of the males were reviewed to ensure that the bird did not sing in response to the song presentation, as this could lead to a different pattern of ZENK expression in the brain

than auditory song exposure alone [73, 75]. No males sang. Across both stimulus conditions, birds generally responded to the initiation of song by adopting an upright, alert posture, orienting toward the speaker, and some emitted a few chirps. All recordings were reviewed to determine whether excess background noise was present. Two birds were eliminated from analysis due to the presence of substantial, unanticipated noise near the testing room. A few other animals were eliminated from analysis of individual brain regions due to histological artifact. Final sample sizes are indicated in the figures.

Tissue Processing

Brains were coronally sectioned at 20µm and thaw mounted onto SuperFrost Plus slides (Fisher Scientific, Hampton, NH) in 6 series. Tissue was stored at -80°C until further processing. One series of slides was processed using immunohistochemistry for ZENK. Tissue was run in three groups due to the large number of slides; both sex and stimulus conditions were equally represented in each. Tissue was warmed to room temperature then rinsed in 0.1M phosphate-buffered saline (PBS). It was then fixed in 4% paraformaldehyde, rinsed 3x5 minutes with PBS, and treated for 30 minutes with 0.9% H₂O₂ in methanol. It was rinsed with PBS, and incubated in 5% normal goat serum in PBS with 0.3% Triton X-100 for 30 minutes. Next, it was incubated overnight in a ZENK (Egr-1) rabbit polyclonal antibody (0.5µg/ml, sc-189, Santa Cruz Biotechnology, Inc., Dallas, TX) in 5% normal goat serum in PBS with 0.3% Triton X-100 at 4°C. The tissue was rinsed in PBS and exposed to a biotin-conjugated goat anti-rabbit secondary antibody (0.5 µg/ml; Vector Labs, Burlingame, CA) in PBS with 0.3% Triton X-100 for 2 hours at room temperature. Following PBS rinses, it was incubated in Elite ABC reagents (Vector Labs, Burlingame, CA) for 1 hour, washed with PBS and Tris-buffered saline and then treated with diaminobenzadine in tris-buffered saline with 0.003% H₂O₂ to produce a brown reaction product. The reaction was terminated with PBS, and the tissue was dehydrated and coverslipped with DPX (Sigma–Aldrich, St. Louis, MO).

An adjacent series was stained with thionin to allow confirmation of the location of the brain regions of interest: NCM, CMM, Tn, lateral and medial striatum, HVC, and LMAN. The auditory cortical regions NCM and CMM were selected due to their role in song learning and perception. The telencephalic song control nuclei, striatum, HVC and LMAN, were selected due to their role in song learning and song production. Tn was selected because it is involved in motivated behaviors in birds [129], and on initial inspection of the tissue it showed high levels of ZENK expression.

Analysis of tissue sections was conducted by an observer blind to treatment condition and sex, using ImageJ software (National Institutes of Health). Each brain region was assessed bilaterally in two adjacent sections in each animal. For all brain regions assessed, a cell was considered labeled if it contained a round nuclear area densely filled with brown stain which was darker than the general background coloring seen in surrounding areas. For NCM, a 0.525mm*0.393mm box was placed with the medial corner under the hippocampus at the point where the ventricle begins to curve ventrally to run parallel with the midline (Figure 3C). A grid of 0.066mm*0.065mm rectangles existed within the box, and cells were counted in alternating cells of the grid excluding cells that overlapped with the bottom or left edge of each grid box. Density was determined by dividing the total number of labeled cells by half of the total area of the region analyzed. Cells within NCM were counted in the section prior to the start of RA and the first section containing RA. For CMM, a 0.496mm*0.205mm box was placed under the ventricle lateral to where it curves ventrally toward the midline between A 1.6 and A 1.2 from a songbird brain atlas [131] (Figure 4E). Area X is located in the lateral striatum of males, but is typically not visible in females [132]. Initial observations indicated substantial differences in the patterns of ZENK expression between the medial and lateral striatum. A box of 0.492mm*0.492mm was placed in the lateral portion of the medial striatum. For the medial striatum, a box of the same size (0.492mm*0.492mm) was placed half way between the midline and the location quantified in the lateral striatum (Figure 5A). Labeled cells were counted in

striatum sections starting in the 4th section after the appearance of LMAN in order to maintain a landmark that was visible in both sexes. For Tn, a 0.238mm*0.244mm box was placed near the ventral edge of the telencephalic lobe where a corner is formed by the ventral and medal edges of the lobe (Figure 6C). As with NCM, cells in Tn were counted in the section prior to the start of RA and the first section containing RA.

Limited labeling was detected in HVC and LMAN, so it was not quantified. However, a qualitative analysis was conducted, in which the areas were observed bilaterally in two adjacent sections and assigned a score from 0 to 2. Zero indicated no labeled cells within the nucleus, 1 indicated very sparse staining or staining that was very light in color, and 2 indicated dark labeling or dense populations of labeled cells within the nucleus.

Statistics

Separate two-way ANOVAs were computed for NCM, CMM, and Tn to determine whether rhythmicity of the stimulus and sex influenced the density of ZENK-immunolabeled cells within the region. A mixed model ANOVA was used for the striatum to assess whether rhythmicity and sex (between animals), as well as location within the striatum (within animals), influenced the density of ZENK-immunolabeled cells. To investigate an interaction, paired ttests were conducted within each sex for lateral and medial striatum. All statistics were calculated using SPSS 21 (IBM, Armonk, NY).

Results

In NCM, a significant main effect of stimulus condition was found ($F_{1,31}$ =5.73, p=0.023), such that the density of ZENK-immunolabeled cells was greater in birds exposed to arrhythmic than rhythmic song (Figure 3). There was no effect of sex on density of ZENK labeled cells ($F_{1,31}$ =0.09, p=0.765), and no significant interaction between stimulus condition and sex ($F_{1,31}$ =0.02, p=0.885).

A significant effect of stimulus condition was also detected in CMM ($F_{1,31}$ =4.34, p=0.046). As in NCM, birds exposed to arrhythmic song had an increased density of ZENKimmunolabeled cells. A main effect of sex was also detected in CMM ($F_{1,31}$ =4.55, p=0.041), such that females had greater density of ZENK-immunolabeled cells than males (Figure 4). A significant interaction between rhythm condition and sex was not detected ($F_{1,31}$ =0.68, p=0.414).

In Tn, a significant main effect of stimulus type was found ($F_{1,31}$ =4.64, p=0.039; Figure 6). As in NCM and CMM, birds exposed to arrhythmic song had greater density of ZENK-immunolabeled cells (Figure 6). There was no effect of sex ($F_{1,31}$ =0.11, p=0.737) and no interaction between rhythm condition and sex ($F_{1,31}$ =0.29, p=0.596). Data for individual groups are presented in Table 1.

Unlike the other areas quantified, the striatum did not show differences between the two stimulus types – ZENK expression was equivalent across birds exposed to normal and arrhythmic songs ($F_{1,30}$ =0.23, p=0.633). There was also no significant effect of sex ($F_{1,30}$ =0.39, p=0.537). However, a significant difference existed between the lateral and medial striatum ($F_{1,30}$ =92.70, p<0.001), and this relationship was affected by sex (sex x location interaction: $F_{1,30}$ =11.94, p=0.002). Expression in both males and females was greater in the medial than lateral striatum (t_{16} =7.972, p<0.001and t_{16} =-5.514, p<0.001 respectively), but the difference appeared much larger in males, largely due to a near absence of labeling in the lateral striatum (Area X) of males (Figure 5). There was no significant interaction between location within the striatum and stimulus condition ($F_{1,30}$ =0.54, p=0.470), nor was there an interaction between sex and stimulus condition ($F_{1,30}$ =0.29, p=0.597). The three-way interaction among sex, location in the striatum, and stimulus condition was also not statistically significant ($F_{1,30}$ =1.43, p=0.241).

For LMAN and HVC qualitative scoring was done in which birds were assigned a number on a 0-2 scale, with 0 indicating no detectable ZENK, 1 indicating light or very sparse labeling, and 2 indicating dark and/or abundant staining comparable to that seen in NCM. In

both LMAN and HVC, scores were very similar across the sexes and stimulus conditions and were mostly 0s (Table 2). No animals were assigned 2s for either brain region (Figure 7).

Figure 3. Density of ZENK expressing cells in NCM. Panels A and B depict representative samples of ZENK immunohistochemical labeling in birds exposed to rhythmic (A) or arrhythmic (B) song. Panel C depicts an adjacent section stained with thionin; the box indicates the area where cells were counted. Panel D shows the density of ZENK expressing cells between sexes and stimulus types (mean \pm standard error). There was a significant main effect of sex, indicted by an asterisk. Sample sizes are noted within the bars.



NCM

Figure 4. Density of ZENK expressing cells in CMM. Panels A-D depict representative samples of ZENK immunohistochemical labeling in a female exposed to rhythmic song (A), female exposed to arrhythmic song (B), male exposed to rhythmic song (C), and a male exposed to arrhythmic song (D). Panel E depicts a thionin stained adjacent section; the box indicates the area where cells were counted. Panel F depicts the density of ZENK expressing cells between sexes and stimulus types (mean ± standard error). A significant main effect of stimulus type is indicated by the asterisk. A significant main effect of sex is represented by the different lower case letters. Sample sizes are noted within the bars.


Figure 5. Density of ZENK expressing cells in the striatum. Panel A depicts a thionin stained section, with boxes showing the lateral and medial areas in which cell densities were assessed. Panel B is from a representative male exposed to rhythmic song, and C is from a female exposed to arrhythmic song. Panel D depicts the density of ZENK expressing cells between sexes, stimulus types, and location within the striatum (mean ± standard error). A main effect of location is indicated by the asterisk. A significant sex x region interaction was also detected, such that the difference in density of ZENK expressing cells in the medial compared to lateral striatum was greater in males than females. Sample sizes are noted within the bars.





Figure 6. Density of ZENK expressing cells in Tn. Panels A and B depict representative samples of ZENK immunohistochemical labeling in birds exposed to rhythmic (A) or arrhythmic (B) song. Panel C depicts a thionin stained adjacent section; the box indicates where cells were counted. Panel D shows the density of ZENK expressing cells between sexes and stimulus types (mean ± standard error). A significant main effect of sex is indicted by the asterisk. Sample sizes are noted within the bars.



Table 1. ZENK+ cells/mm² means (standard error) for the caudomedial nidopallium (NCM), caudomedial mesopallium (CMM), and nucleus taeniae (Tn). The sample size is indicated on the second row of each cell.

		М	ale	Fer	nale
		Left	Right	Left	Right
NOM	Rhythmic	131.7 (23.5) 9	120.6 (26.3) 9	109.9 (24.4) 9	114.4 (22.0) 9
NCIVI	Arrhythmic	194.7 (33.9) 9	205.0 (38.2) 9	162.1 (47.7) 8	218.7 (45.0) 8
CMM	Rhythmic	307.6 (70.1) 9	323.3 (74.6) 9	401.0 (86.3) 9	486.8 (104.7) 9
Civilvi	Arrhythmic	444.2 (92.4) 9	429.5 (105.3) 9	678.6 (156.0) 8	791.1 (153.4) 8
To	Rhythmic	121.5 (34.8) 9	160.7 (41.4) 9	218.1 (32.1) 9	289.9 (50.5) 9
	Arrhythmic	262.1 (64.0) 9	217.2 (97.4) 9	229.2 (53.7) 8	271.2 (43.8) 8

Figure 7. Relative absence of ZENK expressing cells in LMAN and HVC. Panels A and B depict thionin stained sections, with arrows showing borders of LMAN and HVC from a male exposed to arrhythmic song. Panels C and D depict adjacent sections with representative samples of immunohistochemical labeling. LaM = lamina mesopallialis; V = ventricle.



Table 2. Numbers of animals of each sex and stimulus condition exhibiting no or very modest labeling in two cortical song control regions, LMAN and HVC.

		LN	IAN	H	VC
	Score*	Rhythmic	Arrhythmic	Rhythmic	Arrhythmic
Mala	0	7	7	5	7
IVIAIE	1	2	2	4	3
Тс	otal	9	9	9	8
Fomolo	0	8	6	5	3
remale	1	1	2	2	1
Тс	otal	9	8	7	4

*0 = no detectable ZENK expression; 1 = very light or sparse labeling; 2 = dark or dense labeling (no individuals were assigned this score)

Discussion

Summary

The present results indicate that arrhythmic song induces greater ZENK expression in the auditory cortical areas, NCM and CMM, and the amygdala homolog, Tn, compared to unmanipulated (rhythmic) zebra finch song. Effects of stimulus type were not observed in Area X, LMAN or HVC, indicating that these differences in neural responses to song rhythmicity in the adult zebra finch are specific to the regions described. Effects associated with the sex of the animals were detected in two brain areas. First, greater ZENK expression was induced in CMM in females compared to males across stimulus groups. Second, while labeling across the sexes was increased in the medial compared to lateral striatum, the difference was greater in males due to a near absence of ZENK expression in Area X following both types of song stimuli. NCM and CMM

The results in NCM and CMM of increased ZENK expression with arrhythmic as compared to rhythmic song can be considered in the context of human auditory processing. In humans the auditory association cortex has increased activity in response to unexpected perturbations in one's own speech [133]. Neurons in this area are thought to code for mismatch between expected and perceived auditory feedback [133, 134]. The pattern of results seen in NCM and CMM is consistent with data from humans, in which fMRI revealed greater activity in the secondary auditory cortex with exposure to an arrhythmic compared to a rhythmic tone sequence [135]. Thus, one possibility is that the increase in neural activity in NCM and CMM in response to arrhythmic song stems from detection of deviation from the temporal regularity expected based on the learned song template. Data on comparisons of conspecific to heterospecific song are consistent with this idea. Songs from birds other than zebra finches produce little or no expression of ZENK in both NCM and CMM, whereas conspecific song produces a robust response in these regions [43, 44]. We suggest that arrhythmic song is similar enough to natural zebra finch song so as to be detected as a (perhaps inappropriate)

variant of conspecific song, whereas heterospecific song is different enough that it does not activate this system of error detection. It will now be important to determine whether auditory template formation during development is necessary for zebra finches to be sensitive to the rhythmic characteristics of conspecific song.

Exposure to reverse zebra finch song has been used as a way of testing neural response to changes in temporal pattern [136], because the total amount of song and the spectral qualities remain unchanged from normal conspecific song. However, reverse song differs from normal vocalizations in more characteristics than rhythmicity, including the onset and decline within each note and the overall structure of the bout. Reverse song induces less neural activation than other forms of conspecific song in some populations of cells within NCM [42]. These results indicate that the aspects of song altered by reversing it, including timing, bout structure and individual note dynamics, are important for neural responses to conspecific song within NCM. Thus, one possibility is that reverse song is different enough that it is not recognized as an altered form of conspecific song, and does not activate cells within NCM involved in error detection.

Untutored zebra finch song has also been used as a control stimulus; it is produced by a zebra finch and thus has similar motif structure to that of tutored song, but contains notes of unusual frequency, duration, and inflection [36]. Untutored song induces less ZENK expression than tutored zebra finch song in NCM and CMM in both juveniles [76] and adults [86]. These results differ from the current study in that aberrant song reduced ZENK expression rather than increasing it. Similar to the response to reverse song, this pattern may be due to untutored song being too dissimilar to normal song, or too inconsistent, to be detected as normal song with errors. Collectively, the results also indicate that response of auditory cortical neurons requires not just the overall motif and bout structure, but the characteristics of individual notes must be consistent with tutored zebra finch song.

While various auditory stimuli can induce different patterns of ZENK expression in the songbird brain, it is unknown whether these stimuli activate the same types of neurons. The phenotype of ZENK expressing cells was not evaluated in this study. For example, a substantial proportion of the cells in NCM are GABAergic, and these inhibitory cells can influence auditory perception [137]. Increased neural activity within NCM in response to arrhythmic compared to rhythmic song may reflect inhibitory processing rather than stimulation of a functional response. The phenotype of the ZENK+ cells should be evaluated in future studies.

It has been proposed that the auditory song template learned by juvenile zebra finches is stored in NCM [83]. This hypothesis is supported by the finding that in the template formation stage, playback of tutor song induces neuronal activity within NCM and CMM, but not other song system nuclei [138]. Song template storage within NCM is consistent with the hypothesis that NCM is involved in error detection because the site of template storage is a logical location at which to compare the template and a song example. Storage of the template within NCM would also allow NCM neurons to assess other characteristics of song, in addition to rhythm, that could influence perception of whether a sound is conspecific song and the quality of that song.

In the present study, greater ZENK induction was seen in females compared to males in CMM, specifically. Unlike in the song control system [55], sex differences in morphology of auditory structures have not been extensively described; the borders of these brain regions are not particularly distinct, and qualitatively the structure of the regions appears similar in males and females. NCM and CMM are thought to be involved in analysis of songs for purposes of mate selection in females [139]. CMM in particular is able to discriminate between directed and undirected songs [140], which is necessary for evaluating potential mate directed song quality. The increased neural activity in response to song in CMM may therefore be due to CMM being used by females for analysis of potential mates.

Compared to NCM and CMM, much less research has been conducted regarding factors influencing neural activation and ZENK expression in Tn. A previous study in our lab demonstrated that ZENK expression in the Tn of females paired with males is positively correlated with behaviors indicative of pair bonding, including frequency of clumping with a mate as well as frequency and duration of preening [141]. ZENK expression in Tn is also positively correlated with the number of mount attempts in male house sparrows [129]. In ring doves, ZENK expression in Tn in pair bonded birds is greater than in un-bonded birds following a preference test between a mate and a novel bird [142]. In addition, the level of ZENK expression can be accurately used to predict whether the bird is pair bonded [142]. The amygdala is part of a network that controls social behavior, including sexual, parental, and aggressive behavior, in a broad range of species [143, 144]. It is not known whether the birds in this study formed pair bonds prior to being moved to single sex aviaries; if so, they were physically separated from their mates at that point. One possibility is that the increased activity in response to arrhythmic song may be part of the process of evaluating the song as indicating a poor potential partner. The phenotype of ZENK+ cells within Tn in the present study is unknown. However, given the abundance of GABAergic cells seen in the pigeon Tn [145], it is possible that arrhythmic song causes an increase in activity of inhibitory cells, potentially inhibiting selection of the singer for a mate.

An additional potential interpretation of the pattern of neural activation in Tn is suggested by human fMRI and PET studies. When participants were presented with a variety of nonspeech auditory stimuli, activity in the right basolateral amygdala was positively correlated with ratings of unpleasantness of the auditory stimuli [146]. Blood flow increased bilaterally in the lateral amygdala in response to aversive sounds compared to white noise [147]. Additionally, pleasurable music leading to participants getting "chills" reduced blood flow in the amygdala bilaterally [148]. Together these studies indicate that increased activity in the amygdala is

Tn

induced when auditory stimuli are perceived as aversive. The increased activity in Tn may suggest that arrhythmic song is perceived as aversive by zebra finches. This may be combined with the social interpretation, in that a song perceived as aversive may have greater salience for rejection of the singer as a potential mate.

<u>Striatum</u>

In the striatum, an effect of region was detected, such that ZENK labeling was less dense in the lateral (Area X in males) compared to the medial striatum. Interestingly, this difference was greater in males than in females. These results expand on previous data from our lab in juvenile males in which conspecific and heterospecific song, as well as tones, induced a significantly lower density of ZENK labeled cells in Area X than in the medial striatum [46]. In contrast, labeling was uniform throughout the striatum in young females [46]. The current study found a difference between lateral and medial striatum in females as well as males indicating that differences in these areas in females may develop as animals get closer to maturity. Together, these studies suggest that the medial striatum is involved in processing of auditory stimuli, but not in the aspect of rhythmic discrimination assessed in this study. In addition, the low level of ZENK expression in Area X in males has been suggested to indicate a role for this brain region in song learning or production [46] rather than auditory processing, in contrast to the conclusions from some human data [135].

Methodological Considerations

HVC and LAMN. Little ZENK expression was seen in either HVC or LMAN in any of the groups in the present study. While intriguing, these results do not completely exclude the possibility of neuronal activity in response to auditory stimuli in these two regions. In fact, HVC exhibits specific electrophysiological responses to a bird's own song [89] and its tutor's song [90], with much lower responses to general conspecific song [90]. These results are consistent with the present data which showed limited ZENK expression in these regions in response to the songs of unfamiliar zebra finches. In addition, one needs to consider that analyses of ZENK

protein and electrophysiology do not always show the same pattern [42, 85]. It has been proposed that immediate early gene expression may be regulated differently in several nuclei, including HVC and LMAN, than the rest of the brain because ZENK is not expressed in these areas after presentation of stimuli that induce electrophysiological responses [74] or after treatment with a GABA antagonist [149]. Thus, while this study does not suggest a role for these areas in rhythm processing, the possibility cannot be rejected based on the present data.

Stimuli. Sound levels in the intervals between syllables were not identical between the rhythmic and arrhythmic stimuli. However, these differences are highly unlikely to have affected our results for several reasons. First, no significant correlations were detected between ZENK labeling and the average intensity of the intervals between syllables in either the rhythmic or arrhythmic group for any of the regions that showed an effect of stimulus type (all r<0.37, p>0.14). Second, the average power of these intervals was less than 1.2% of that of the syllables for both manipulations. Characteristics of these gaps between syllables other than their duration are therefore probably far less salient than the notes themselves. Third, the difference between the power levels of the intervals in the two stimulus types as measured in playback through the speakers is half of that in the pure stimuli (which are depicted in Figure 2). Finally, the power of the intervals was not consistently higher in either the rhythmic or arrhythmic stimulus.

Potential Translational Implications

The zebra finch has been used previously as a model for developmental stuttering. Delayed auditory feedback can induce stuttering like syllable repetitions in zebra finches [150, 151] indicating the importance of normal auditory feedback for accurate vocalization. Helekar et al. (2003) found that 7% of the males in their colony naturally produce a stuttering-like song with single syllable repetitions, and 53% of males tutored by these repeaters also produce single syllable repetitions in their song [152]. Based on fMRI data, these birds that learn to repeat syllables have decreased responses to tutor song and increased responses to unfamiliar

conspecific song in field L [153], the avian homolog of the primary auditory cortex [53]. These results suggest some dysfunction in the learning process, perhaps related to storage of an auditory template. However, activity in NCM and CMM was more variable across the syllable repeating and normal song groups, thus significant differences could not be discerned [153]. Assessment of neural responses as stuttering-like song develops would provide further understanding of specific neural mechanisms.

Animal models for many of the other disorders that involve deficits in rhythm and timing perception exist, but in these cases as well the focus is on aspects of the disorders other than timing and rhythm. For example, models of autism center around the presence of social and stereotyped behaviors in rodents [154]. Rodent models of schizophrenia are widely varied with effects on motor, cognitive and social behaviors [155]. A rat model of dyslexia with specific neurological deficits has impairment in tasks of time perception [156], but the rat as a model is restricted in its applicability to communication disorders because this species does not learn complex vocalizations. While valuable information is collected from these models, songbirds offer advantages due to specific similarities to humans. For example, they are vocal learners, undergoing critical periods of auditory and sensorimotor learning to achieve highly stereotyped yet complex adult-like songs. In addition, they rely on visual and auditory cues as opposed to olfactory, and they form monogamous pair bonds. Further study of the basis of rhythm perception and rhythm deficits is needed in animal models in order to begin developing new therapies that target the timing-based deficits observed in this broad range of disorders.

In sum, zebra finches are an excellent potential model for studying neural mechanisms underpinning human rhythm perception and its relation to speech and language processing. This avian species provides a model through which neurochemical mechanisms of rhythm perception and dysfunction can be tested to gain a deeper understanding of rhythm processing for application to both healthy and disordered human development. The present study has shown that NCM, CMM, and Tn increase neural activity in response to arrhythmic song,

indicating a role for rhythm in auditory discrimination and social behavior such as mate choice in the zebra finch. Further studies are needed to understand the development and mechanisms underlying neural responses to rhythm.

CHAPTER 2 Neural Responses to Rhythmicity in Juvenile Male and Female Zebra Finches

Introduction

Evidence is increasing for an association between rhythm skills and language development. For example, in typical speakers, there is a positive relation between language and literacy skills and the ability to analyze beat-based rhythmic sequences [1]. Conversely, children with specific language impairment have difficulties processing and synchronizing tapping with a rhythmic stimulus [5, 6]. People who stutter appear to have deficits in the internal generation of rhythm, and both adults and children who stutter can dramatically reduce the rate of disfluencies when they synchronize their speech with an external rhythmic stimulus such as a metronome [2, 157] or music [4]. Children who stutter have a reduced ability to discriminate complex rhythms as compared to their age-matched peers [12]. Thus, the development of rhythmic processing abilities is important for typical speech and language development. A deeper understanding of how rhythm is processed in the brains of individuals at all stages of development should aid in understanding a range of developmental speech and language disorders, as well as other conditions that involve deficits in rhythm processing such as Parkinson's disease [11].

An appropriate animal model allows for levels of investigation not possible in human subjects, and songbirds such as zebra finches can be particularly useful for the study of vocal development (reviewed in [29]). For example, in both species, vocalizations of adult tutors are memorized to form a template and then juveniles practice vocalizations to make modifications that increasingly match the template. This process includes babbling in humans as well as subsong and plastic song in birds such as zebra finches. Ultimately this process results in adult speech in humans and crystalized song in zebra finches. Vocal learning is limited to a critical period in both species.

It has been theorized that vocal learning is a necessary factor for the capacity to perceive and entrain to rhythmic auditory stimuli [30]. A broad range of species have been identified as having this capacity, including elephants [158, 159], cetaceans [160], bats [161, 162], parrots [163], hummingbirds, and songbirds [29, 53, 164]. Recent studies have indicated that at least one species that does not learn vocalizations has the capacity to entrain to a beat [31, 32]. While the extent to which vocal learning is required for rhythmic entrainment is still unclear [33], a far greater number of vocal-learning species than non-vocal learners have demonstrated a capacity to entrain to a beat [34]. As one of the few of these species amenable to breeding in laboratory conditions, the zebra finch is a good model to study the juvenile development of rhythm perception. Their songs, which are used for nest site defense and courtship, are highly rhythmic [35, 36]. A zebra finch song bout consists of a sequence of repetitive introductory notes, followed by repetitions of an ordered set of syllables called a motif. The intervals between notes in these songs are very regular (Figure 8). The natural rhythmic structure of zebra finch song adds to their value as a model species to improve our understanding of how rhythm perception develops in a vocal-learning species.

ZENK is an immediate early gene frequently used to study neural activity in zebra finches. ZENK is an acronym for the multiple names that have been assigned to this evolutionarily conserved protein, zif-268 [77], egr-1 [78], NGFI-A [79], and Krox-24 [80]. ZENK is involved in learning and synaptic plasticity [82], and can act through regulation of other genes through a DNA binding site [81]. Inhibition of ZENK expression in juvenile zebra finches during tutor song exposure prevents normal song learning [83].

Figure 8. A representative spectrogram of rhythmic song. A natural zebra finch song used to generate one of the stimuli from the present study is depicted. Introductory notes are labeled with I. A, B, and C labels indicate separate syllables in the song, with each repetition of the three syllables constituting a motif. Modified from Figure 1 in [123].



A previous study in our lab focused on rhythm effects on ZENK expression in the adult zebra finch brain [123]. ZENK expression was assessed in the caudomedial nidopallium (NCM), the caudomedial mesopallium (CMM), which while anatomically distinct in the zebra finch brain, are both considered analogous to the auditory association cortex in humans [68], and nucleus taeniae (Tn) which is analogous to the mammalian amygdala [129]. NCM is likely the location where the learned song template is stored in the brain [83, 138, 165]. We hypothesize that this template may contain information about the proper timing of songs, allowing for discrimination of timing and rhythmicity. In our study on adults [123], birds exposed to song that was modified to disrupt its natural rhythmic structure had significantly more ZENK expression in all three brain regions compared to birds exposed to song with the same syllables presented with the original (unmodified) rhythm. These different responses in secondary auditory areas may suggest that birds perceive errors in the arrhythmic song relative to the learned template. The increased activity in Tn, which is involved in pair bonding and mate selection [129, 141, 142], may indicate an aversion to the disrupted rhythm and an assessment of poor quality as a potential mate.

The general timeline of vocal development in zebra finches is agreed upon, but the exact ages at which specific milestones occur is still debated to some degree [47-50]. Template

formation occurs between approximately post-hatching days 20 and 60 [48-50]. In males, vocalizations begin as food begging, then develop into subsong, an immature form of vocalization that is quiet and contains poorly formed notes with greatly variable structure and sequence [36]. As the males mature, these songs are practiced and modified to relatively closely match the learned template. This phase of sensorimotor integration occurs between about post hatching day 25 and sexual maturity which occurs around 90 days of age [48-50]. After sensorimotor integration, males have a crystalized song that will not undergo significant changes throughout the rest of their lives under normal circumstances [47]. In addition to learning to produce specific song syllables, juvenile males also learn a specific grammar to structure their song [166].

Understanding the developmental trajectory of neural responses to rhythm is useful in elucidating their relationship to function. We exposed birds to rhythmic or arrhythmic song prior to and during the template formation period, as well as during early sensorimotor integration. Differences in responses to the two types of stimuli prior to acquisition of a song template could indicate an innate capacity to perceive song-related rhythms. Discrimination during and after template formation, but not before, would suggest that characteristics of rhythm are learned. If rhythm discrimination emerges during sensorimotor integration in males, it would suggest that a motor component is required for distinct neural responses to rhythmicity. Differences between the sexes may be informative here as well, as both males and females appear to form templates [51], even though only males engage in the production of song [36].

Material and methods

Subjects

Zebra finches hatched and were reared in aviaries containing 5-7 adult pairs and their offspring. On day of hatching, toes were clipped as a means of unique identification. Tissue from the toes was used to identify the sex of the birds through polymerase chain reaction [167].

A total of 108 birds were used (18 males and 18 females at 15-17 days post hatching (d15), 25-27 days post hatching (d25), and 45-47 days post hatching (d45)). All animals were maintained on at 12:12 hour light/dark cycle and had ad libitum access to seed (Kaytee Finch Feed; Chilton, WI, USA), water, cuttle bone and gravel. Their diet was supplemented weekly with hard boiled chicken eggs, bread, spinach, and oranges. All procedures were approved by the Institutional Animal Care and Use Committee of Michigan State University.

Stimulus Exposure

Rhythmic and arrhythmic zebra finch song stimuli were used from our study on adults [123]. Details regarding their creation are reported in that paper. Briefly, rhythmic song stimuli were generated from recordings of natural zebra finch song, and arrhythmic stimuli used the same recordings with the silent intervals between notes altered to disrupt the temporal pattern of the song. Three different stimuli of each type were created, each of which contained three of nine different songs that were generated. Each song was presented for 30 seconds followed by 30 seconds of silence, with the three songs repeating in random order for 30 minutes. All songs of each type were novel to all subjects.

Nine males and 9 females of each age group were exposed to each stimulus type (rhythmic or arrhythmic). One bird was exposed at a time between 9am and 3pm. Birds were placed into a sound isolation chamber (252-Mini Sound Shelter, IAC Acoustics, Bronx, New York, USA) to habituate for one hour. Following the habituation period, the stimulus was presented at approximately 70dB. Birds remained in the chamber for one hour following song exposure to allow for peak ZENK protein expression. Brains were then immediately collected and flash frozen in methylbutane.

All song exposures were recorded using a Canon Vixia HF R300 camcorder. Recordings were reviewed to ensure birds were not producing subsong and no extraneous background noise existed that might influence their auditory responses.

Tissue Processing

Brains were sectioned coronally at 20µm and collected in 6 series. Sections were thaw mounted onto SuperFrost Plus slides (Fisher Scientific, Hampton, NH). One set of tissue from each bird was thionin stained to allow for clear identification of anatomy.

An alternate set of tissue from each bird was processed for immunohistochemistry for ZENK. Tissue was processed in five groups due to the large number of slides. All experimental groups were evenly represented in all immunohistochemistry runs. The protocol was conducted as described in [123]. Briefly, slides were warmed to room temperature, fixed 4% paraformaldehyde, and rinsed in 0.9% hydrogen peroxide in methanol. Slides were then incubated in 5% normal goat serum in 0.1M phosphate buffered saline (PBS) with 0.3% Triton X-100. Slides were incubated overnight at 4°C in ZENK (Egr-1) rabbit polyclonal antibody (0.5 μ g/ml, sc-189, Santa Cruz Biotechnology, Inc., Dallas, TX) in 5% normal goat serum in PBS with 0.3% Triton X-100. The next day slides were incubated in biotin conjugated goat anti-rabbit polyclonal antibody (0.5 mg/ml; Vector Labs, Burlingame, CA) in PBS with 0.3% Triton X-100, followed by Elite ABC reagents (Vector Labs, Burlingame, CA), and then treated with diaminobenzidine with 0.003% H₂O₂ to produce a color reaction.

An investigator blind to the stimulus exposure condition, sex, and age of the birds conducted analysis of all slides using ImageJ software (National Institutes of Health, Bethesda MD). All regions of interest were analyzed bilaterally in two adjacent sections. Boxes were placed within NCM, CMM and Tn as described and depicted in [123], and all cells containing a dark brown stained nucleus that was clearly distinguishable from the surrounding tissue were counted. The density of ZENK expressing cells (labeled nuclei per unit area) was calculated for each brain region. Due to high levels of baseline labeling apparent in these juvenile brains, values were also assessed in the control region nucleus rotundus (Rt), a thalamic visual area that should show no ZENK induction specifically from the auditory stimuli. A 0.268mm*0.268mm box was placed in the center of the region bilaterally in two adjacent

sections and density of ZENK expressing cells was calculated. For each animal, average density values were calculated for each region of interest, collapsing across the hemispheres. Data analysis

The brains stained in the final run of immunohistochemistry were excluded from analysis due to unusually high levels of background staining unique to this set of tissue, reducing the total number of animals in each group by one. Occasionally individual brain regions were not able to be analyzed in particular birds due to damage to the tissue sections. Final sample sizes are indicated in Table 3. Compared to our study in adults, constitutive levels of ZENK expression were increased across brain region and ages. Therefore, labeling in Rt was used as a covariate. The density of ZENK+ cells in Rt correlated significantly with the values obtained from NCM, CMM, and Tn (all r > 0.642, p< 0.001). Separate three-way ANCOVAs were run for NCM, CMM, and Tn, with age, sex, and stimulus type (rhythmicity) as factors. To provide pairwise comparisons probing main effects of age detected in NCM and CMM, separate oneway ANCOVAs, with rhythmicity as the factor, were conducted within each region. To investigate interactions of age and stimulus type detected in NCM and CMM, individual one-way ANCOVAs were conducted within each age group, as well as across all ages within each rhythm condition, with further one-way ANCOVAs used to provide pairwise comparisons between each combination of two ages within a rhythm condition. For all sets of pairwise comparisons, a Holm's Bonferroni correction was used [168], with adjusted α -levels indicated with each result. The Holm's correction provides α -values at which to assess the significance of multiple comparisons, with the most significant result analyzed at the most strict α -value of 0.05/total number of comparisons, as in a traditional Bonferroni correction, and further comparisons assessed in rank order of their significance, reducing the value of the denominator in the α equation by one with each comparison. All statistics were calculated using SPSS (IBM, Armonk, NY).

Results

No main effects of sex were detected in NCM ($F_{1.79}$ =1.079, p=0.302), CMM ($F_{1.82}$ =0.288, p=0.593), or Tn ($F_{1.79}=0.341$, p=0.561). However, in NCM, there was a main effect of age ($F_{2.85}$ =3.388, p=0.039; Figure 9). D15 birds had a higher density of ZENK expressing cells than those at d25 ($F_{1.58}$ =8.415, p=0.005, α =0.0167), and a trend existed for greater ZENK expression at d15 compared to d45 ($F_{1.60}$ =4.413, p=0.040, α =0.025). ZENK expression did not differ significantly between d25 and d45 ($F_{1,57}$ =0.960, p=0.331, α =0.050). While a main effect of rhythm condition did not exist ($F_{1.85}$ =0.458, p=0.501), age and stimulus type interacted ($F_{2.85}$ =3.189 p=0.047; Figure 9). At d15, a trend toward a greater density of ZENK+ cells was detected in response to rhythmic compared to arrhythmic song ($F_{1,29}$ =6.038, p=0.020, α =0.0167). However, no effect of the stimulus type was detected at d25 ($F_{1.29}$ =1.383, p=0.250, α =0.025) or d45 ($F_{1,29}$ =0.300, p=0.865, α =0.050). In addition, there was an effect of age within birds exposed to rhythmic ($F_{2,42}$ =6.983, p=0.002, α =0.025), but not arrhythmic ($F_{2,42}$ =0.057, p=0.945, $\alpha=.050$) song. In birds that heard the rhythmic song, a greater density of ZENK expressing cells was observed at d15 compared to both d25 ($F_{1,28}$ =14.023, p=0.001, α =0.0167) and d45 ($F_{1,28}$ =5.784, p=0.023, α =0.025), and in animals at d45 compared to d25 ($F_{1,27}$ =4.817, *p*=0.037, α=.050).

Similarly, a main effect of age was detected in CMM ($F_{2,82}$ =8.446, p<0.001; Figure 10). D15 birds had greater ZENK expression than those at d25 ($F_{1,60}$ =11.900, p=0.001, α =0.025) and d45 ($F_{1,61}$ =13.805, p<0.001, α =0.0167), whereas no difference was detected between birds at d25 and d45 ($F_{1,60}$ =0.544, p=0.464, α =0.050). A main effect of rhythm condition was not seen ($F_{1,82}$ =0.470, p=0.495), but age and stimulus type interacted ($F_{2,82}$ = 3.485, p=0.035; Figure 10). At d25, a greater density of ZENK expressing cells was detected in response to arrhythmic compared to rhythmic song ($F_{1,28}$ =7.061, p=0.013, α =0.0167). However, there was no effect of rhythm condition at d15 ($F_{1,29}$ =1.671, p=0.206, α =0.025) or d45 ($F_{1,29}$ =0.001, p=0.973, α =0.050). In addition, there was an effect of age in birds exposed to both rhythmic ($F_{2,43}$ =7.788, p=0.001,

 α =0.025) and arrhythmic ($F_{2,44}$ =3.397, p=0.042, α =0.050) song. In those hearing rhythmic song, an increased density of ZENK+ cells was observed in d15 compared to d25 ($F_{1,28}$ =17.591, p<0.001, α =0.0167), but no differences were seen between d15 and d45 ($F_{1,29}$ =2.928, p=0.098, α =0.050), or in animals at d25 compared to d45 ($F_{1,28}$ =3.678, p=0.065, α =0.025). Within the birds exposed to arrhythmic song, a trend was detected toward greater ZENK expression in birds at d15 compared to d45 ($F_{1,29}$ =6.016, p=0.020, α =0.0167), but no differences between d15 and d25 ($F_{1,29}$ =0.787, p=0.382, α =0.050) or d25 and d45 ($F_{1,29}$ =3.673, p=0.065, α =0.025) were seen.

Main effects of rhythm and age were not detected in Tn ($F_{1,79}$ =0.021, p=0.884; $F_{2,79}$ =0.992, p=0.376 respectively), and no age by rhythm interaction existed ($F_{2,79}$ =1.617, p=0.205) (Table 3). **Figure 9. Density of ZENK expressing cells in NCM.** The photographs depict representative samples of ZENK expression in males at A) d15 exposed to arrhythmic song, and males exposed to rhythmic song at C) d15, D) d25, and E) d45. Panel B depicts the density of ZENK expressing cells across ages and rhythmic conditions. Marginal means from the overall ANCOVA were used to graph adjusted means ± SEM. Group sizes are listed within the bars. Data are collapsed across sexes, as no significant effects were detected. A main effect of age was detected, with greater density of ZENK expressing cells at d15 compared to d25 and a trend for greater expression at d15 compared to d45. Age and stimulus type (rhythmicity) also interacted. Differences between ages within the birds exposed to rhythmic song are indicated by different bold letters within the bars. # = Trend toward an increase in response to rhythmic song at d15.



Figure 10. Density of ZENK expressing cells in CMM. Photographs depict representative samples of ZENK expression in males exposed to rhythmic song at A) d15, and C) d25, and D) a male exposed to arrhythmic song at d25. Panel B indicates the density of ZENK expressing cells across ages and rhythmic conditions. Marginal means from the overall ANCOVA were used to graph adjusted means ± SEM. Group sizes are listed within the bars. Data are collapsed across sexes, as no significant effects were seen. A main effect of age was detected, such that a greater density of ZENK expressing cells was present a d15 compared to both d25 and d45. Effects of age and stimulus type (rhythmicity) also interacted. Differences between ages within birds exposed to rhythmic song are indicated by the bold letters within the bars. # = a trend for an increase at d15 compared to d45 within birds exposed to arrhythmic stimuli. * = Significant increase in response to arrhythmic compared to rhythmic song at d25.



CMM

Table 3. Unadjusted ZENK+ cells/mm² means and SEM for the caudomedial nidopallium (NCM), the caudomedial mesopallium (CMM), and nucleus taeniae (Tn). Values within each cell indicate the mean with the standard error in parentheses and n on the row below.

		d1	5	d2	5	d4	5
		Male	Female	Male	Female	Male	Female
	Rhythmic	671.8 (92.3) 9	791.7 (55.2) 9	387.6 (104.3) 9	334.6 (128.7) 9	430.6 (153.3) 8	273.2 (83.1) 9
	Arrhythmic	630.0(131.7) 9	629.5 (109.6) 9	481.7 (117.0) 8	394.9 (86.5) 8	351.6 (107.3) 9	352.8 (117.4) 9
	Rhythmic	1063.6 (132.6) 9	944.4 (128.8) 9	560.1 (143.0) 9	485.0 (150.5) 9	430.0 (151.7) 9	428.9 (147.4) 9
CIMIN	Arrhythmic	973.9 (158.0) 9	1020.3 (180.9) 9	561.3 (77.8) 9	765.8 (147.2) 9	538.4 (144.4) 9	513.9 (158.8) 9
s F	Rhythmic	840.4 (158.1) 9	864.8 (84.8) 9	592.0(126.1) 9	532.9 (123.0) 9	558.7 (163.4) 8	535.6 (170.3) 9
Ξ	Arrhythmic	749.4 (169.6) 9	952.1 (168.0) 9	681.6 (112.2) 8	641.2 (99.0) 8	421.1 (97.9) 9	586.2 (126.0) 9

Discussion

Summary of Specific Effects

Expression of the immediate early gene ZENK across the two sexes was affected by developmental stage and rhythmicity of song stimuli in two auditory regions of the zebra finch brain, NCM and CMM. In contrast, neither variable influenced the density of ZENK expressing cells in Tn, a homologue of the mammalian amygdala. Several results were parallel across the two auditory regions. For example, expression was greater at d15 compared to d25 in both NCM and CMM and at d15 compared to d45 in CMM; a trend for this age difference also existed in NCM. Similarly, within birds exposed to rhythmic stimuli, increased ZENK in both regions was detected at d15 compared to d25, effects that were not detected in either area in birds exposed to the arrhythmic songs. The magnitude of other effects differed between the NCM and CMM, although the patterns were quite similar across the two regions (compare figures 9B and 10B). Specifically, in NCM only, the density of ZENK+ cells in birds exposed to rhythmic song was greater at d15 than d45 and at d45 than d25, whereas in CMM ZENK was only increased in d15 compared to d25 in birds that heard the rhythmic stimuli. While significant differences between pairs of ages were not detected in either cortical region, a trend was detected for an increase at d15 compared to d45 in birds exposed to the arrhythmic stimulus. Finally, effects of stimulus type within specific ages differed across the two regions, such that at d25 arrhythmic song resulted in greater ZENK expression in CMM but not NCM, and a trend for an increased response to rhythmic song existed at d15 in NCM but not CMM.

The increased ZENK expression generally detected in the youngest birds in this study parallels results from work documenting relatively high levels of constitutive expression of this protein at post-hatching day 20 in both NCM [42, 92] and the broader auditory lobule [169]. These earlier studies suggested that ZENK was not inducible with conspecific song in these regions. The present work cannot address this issue specifically, but using expression in Rt as a covariate allows us to conclude that ZENK is induced in NCM in response to typical song at

d15 and to a greater degree than at later developmental stages. The lack of age and rhythm effects in Tn indicate that the effects detected in NCM and CMM are specific to these auditory areas, and not indications of global increases in activity or constitutive high ZENK expression in the early juvenile period.

In both NCM and CMM, the overall effect of age appears to be driven by data from birds that heard rhythmic song, suggesting that developmental changes in auditory processing occur primarily in natural stimuli. The substantial reduction between d15 and d25 in ZENK in response to rhythmic song in both regions could be related to the birds' focus on memorizing song from tutors at the later age rather than reacting to the novel vocalizations presented in the current study. ZENK expression is associated with synaptic plasticity and learning [82], and during template formation substantial synaptic changes would be required for acquisition of the tutor song memory.

<u>NCM</u>

The trend we detected toward greater ZENK expression in response to rhythmic than arrhythmic song in NCM is consistent with other data collected during song development suggesting an innate preference toward more natural, species typical sounds. For example, during the critical period for template memorization, birds reared in isolation show a behavioral preference for conspecific over heterospecific song by landing more frequently on a perch that elicits playback of conspecific song [170]. Additionally, juvenile zebra finches that have not been exposed to adult song prefer songs with more common elements, as shown by time spent near a speaker [171].

The pattern of ZENK expression in response to the same auditory stimuli used in the present study differed in the NCM of adult zebra finches. In the older birds, greater ZENK expression was detected in individuals hearing arrhythmic compared to rhythmic song [123]. Such a difference across ages is not surprising, as substantial development occurs within NCM in the late juvenile period. For example, estrogens synthesized within NCM become sexually

dimorphic between 46 and 80 days post hatching, with greater estrogen levels present in males compared to females [172]. Estradiol facilitates improved discrimination of auditory stimuli within NCM [108-111]. While effects of this hormone on rhythm perception in songbirds are unknown, it is possible that adult responses may require levels of estradiol availability not present in the juvenile birds in the present study.

It is also possible that relative levels of habituation influenced the ZENK responses in juveniles and adults. Faster habituation of electrophysiological responses to songs is seen in NCM in 35 day-old compared to adult zebra finches [173]. Reduction of the ZENK response following repeated presentations of auditory stimuli also occurs in adult zebra finches [174]. While the ZENK expression in juvenile birds exposed to repeated presentations of the same stimulus has not yet been assessed, it is possible that the pattern of increased habituation of neural responses to auditory stimuli, including those in the present study, may result in reduced ZENK expression within the juvenile NCM relative to its potential peak level, which could result in diminished ability to detect differences in ZENK expression to the different stimuli.

A third possible explanation for the different pattern seen between d15 and adult birds [123] relates to the phenotype of the cells. Many GABAergic neurons are present in NCM, and some of them express ZENK in response to song in adult birds [137, 175]. The GABAergic neurons in NCM are involved in maintaining the selectivity tutor song responsive neurons, and in the late juvenile period the inhibition of GABAergic cells can allow tutor song selective neurons to fire in response to a broader range of song stimuli [165]. In zebra finches 51-83 days post-hatching, injection of GABA agonists into the auditory nucleus NIf is sufficient to disrupt the rhythm and stereotypy of plastic song [176]. Together these results indicate that by late in juvenile development GABAergic neurons are active within the auditory system and are important for song control and specificity of auditory responses. It is possible that in the present

study the birds were too young for the inhibitory network of GABAergic neurons to be sufficiently developed to facilitate auditory discrimination.

<u>CMM</u>

The pattern of ZENK expression at d25 in CMM was the same as we detected in adults, with arrhythmic song producing an increase compared to rhythmic song [123]. That this pattern was detected at d25 during template formation as well as in adulthood, but not at d45 during sensorimotor integration in males, leads to a challenge for interpretation. One possibility is that birds are focused around d25 on accurately perceiving external stimuli for creation of the highest fidelity match and this focus on auditory perception is important again in adulthood when the quality of a song is important for mate attraction and nest site defense. In contrast, during sensorimotor integration around d45, male zebra finches are more focused on the motor task of matching their own vocalizations to a preexisting template. The zebra finch brain may have a greater capacity to discriminate auditory stimuli during times when primary attention toward auditory signals is ecologically relevant, and both sexes may show a reduced capacity to discriminate rhythms at ages important for sensorimotor integration in males. If this explanation is valid, it is unclear why the CMM of females would not exhibit differential responses to the two song types at d45, as they do not practice vocalizations. One possibility is that while males at this age are focused on sensorimotor integration, females may undergo a different developmental process that may reduce their attention toward auditory discrimination. It is also possible that the developmental trajectories are parallel without obvious functional consequences for females.

Similar to the pattern we saw in CMM across d25 birds in the present experiment and our study on adults [123], electrophysiological responses within NCM differed across a range of song stimuli at d20 and in adulthood, but not at d35 [173]. While this effect was found in NCM rather than CMM, it is evidence that response patterns within the brain emerge during early stages of development, regress in later stages and are present again in adulthood.

In the present study, no effect of sex was seen in CMM in juvenile zebra finches which contrasts with adult zebra finches in which ZENK expression was enhanced in females compared to males across the same auditory stimuli [123]. The higher ZENK expression in CMM in adult females may be due to involvement of CMM in evaluating the quality of a male's song for the purpose of mate selection which would not occur prior to sexual maturity.

<u>Tn</u>

The differences in results in Tn compared to the adult study [123] may also relate to sexual maturity. While no effects of age or rhythm were seen in this region in the present study on juveniles, a greater density of ZENK expressing cells was observed in the Tn of adult birds that heard arrhythmic compared to rhythmic song [123]. Populations of cells in this brain region in adult Bengalese finches are also responsive to particular types of vocalizations such as song or calls generated by only males or females [177], suggesting its importance in processing socially relevant stimuli. Tn appears to be involved in mate selection and pair bonding; it responds in multiple avian species to acts of mating or the presence of a partner, and can indicate the strength of a pair bond [129, 141, 142]. As for the other regions in this experiment, it will eventually be important to investigate responses to differences in the rhythmicity of song during the transition into adulthood.

Conclusions

Overall, the results of the present study, based on relative densities of cells expressing ZENK protein, suggest that the ability to discriminate temporal regularity in auditory stimuli fully develops between the stages of sensorimotor integration and adulthood. Some degree of innate rhythmic discrimination may exist in NCM, but the pattern of responses to rhythmic vs. arrhythmic stimuli is reversed in adults compared to 15 day-old birds [123], leaving the possibility that there is also a learned component, as error detection in arrhythmic song hearing adults could be facilitated by memorization of rhythmic regularity as a component of the song template. In CMM, a learned component to rhythm discrimination is likely since differences in

ZENK expression first appear during template formation. The development of rhythm discrimination is a complex process that develops at different rates across these brain regions. As in the present study, most research has focused on zebra finch neural development during the time periods surrounding major milestones in the development of vocal learning. Further work is necessary to understand how the auditory responsive brain nuclei mature during the late juvenile period in order to provide adult responses to varied stimuli.

CHAPTER 3

ZENK Induction in the Zebra Finch Brain by Rhythmic and Arrhythmic Song: Relationship to Estradiol and Sex

Introduction

Estradiol (E2) influences perceptual systems in a large range of animals. The hormone may act at both peripheral and central levels. For example, estrogen receptor α is present in the retina [94-96], and estrogen receptors α and β are present in many sensory organs, including the olfactory epithelium [93], dorsal root ganglion, as well as the cochlea [97-99] in diverse species. Women with high levels of E2 show attenuation of auditory event-related potentials in the cortex [103], and auditory evoked responses shift more left lateralized during high E2 phases of the menstrual cycle [178]. Auditory brainstem responses are also influenced by E2 levels in rats [104], rhesus monkeys [105], and humans [106].

In zebra finches, the caudomedial nidopallium (NCM), a secondary auditory cortical region, and nucleus taeniae (Tn), the avian homolog of the mammalian amygdala, are sites of substantial E2 synthesis and activity [119, 179]. Both areas have abundant expression of aromatase [114-116], the enzyme responsible for the metabolism of E2 from testosterone. NCM, Tn, and to a lesser degree the caudomedial mesopallium (CMM), another secondary auditory region, express both estrogen receptor α [117, 118] and the membrane bound receptor GPR30 [120]. Microdialysis in NCM of awake behaving zebra finches reveals a significant increase in E2 concentration during exposure to conspecific song in both males [122] and females [108].

Effects of E2 on auditory discrimination in songbirds have been assessed through multiple methodologies. Electrophysiological recordings within the zebra finch NCM with simultaneous retrodialysis of E2 showed consistently increased neural responses to conspecific songs [107-109]. The pattern is complicated, however, by increased responses to white noise detected in some studies but not others [107-109], and retrodialysis of fadrozole (a potent

aromatase inhibitor) reducing overall responses to sound in one study, but not another [108, 109] [180]. Thus, while E2 within NCM seems to increase responses to conspecific song, its exact influence on auditory selectivity in this region is unclear. In seasonally breeding white-throated sparrows, no differences exist in expression of the immediate early gene ZENK in the NCM or CMM of non-breeding individuals between exposure to conspecific song or tones. However, with systemic E2 replacement higher ZENK is seen in birds exposed to song [110]. These effects are not due to an increase in ZENK in response to conspecific song, but instead to a decrease in response to tones [110]. Similar patterns of ZENK expression are seen in brain regions within the social behavior network [111] including Tn, where birds in the breeding season have greater ZENK expression in response to song than to tones or silence, and exogenous E2 induces this difference in non-breeding birds [111]. Together these studies suggest a strong role for E2 in auditory processing in songbirds.

A growing body of evidence links rhythmic ability to linguistic skills in humans. In young adults, a positive correlation exists between the ability to process rhythmic sequences and language and literacy skills [1]. In addition, deficits in rhythm processing have been detected in a range of speech and language disorders such as dyslexia [10], stuttering [12], and specific language impairment [5, 6]. Elucidating the factors that influence neural processing of rhythm should aid in understanding these and other disorders involving rhythmic processing deficits. As a vocal learning species with a naturally rhythmic song [35], the zebra finch is a strong model to investigate rhythm processing.

Previous research from our group has investigated the effect of the rhythmicity of song on ZENK responses in NCM, CMM and Tn [123]. In all three regions, ZENK expression was increased in adult birds exposed to song modified to disrupt the natural temporal structure (arrhythmic) compared to song with the timing unaltered (rhythmic) [123]. Considering data from humans [133-135], these results were interpreted as the increased activity in NCM and CMM potentially indicating errors in arrhythmic song relative to the more natural vocalizations.

Increased activity in Tn may reflect an indication of a poor potential mate in this region [141, 177].

In order to further understand the mechanisms underlying auditory rhythm discrimination, here we investigated potential impacts of E2 on neural ZENK responses to rhythmic and arrhythmic song stimuli by both increasing and decreasing E2 availability.

Methods

<u>Subjects</u>

Zebra finches (54 male and 54 female) hatched and were raised in walk-in aviaries, housing 5-7 pairs of adult birds and their offspring. Birds were kept on a 12:12 light:dark cycle, and given ad libitum access to water, seed (Kaytee Finch Feed; Chilton, WI, USA), gravel, and cuttlebone. The birds received weekly dietary supplementation with spinach, oranges, bread, and hard boiled chicken eggs. When birds were a minimum of 90 days of age they were transferred to walk-in single sex aviaries housing 30-60 birds and allowed to acclimate to their new housing conditions for at least 10 days. Birds remained in the single sex aviaries until the start of hormone manipulation. All procedures were approved by the Institutional Animal Care and Use Committee of Michigan State University.

Hormone Manipulation

Three groups were created: circulating E2 was (1) increased via implants and (2) decreased using fadrozole injections; (3) control birds were treated with appropriate vehicles. Since fadrozole is water soluble and is commonly administered via injections in a saline vehicle, and E2 is lipid soluble and most easily administered via long-term treatment in Silastic capsules [181], we gave each group both an implant and daily injections in order to ensure all treatment groups experienced the same manipulations. As in [86], E2 capsules were created by packing 2mm of 17β -E2 (Steraloids, Newport, RI) into a 5mm length of Silastic tubing and sealing the ends with silicone. Blank pellets were sealed without packing with hormone. Birds were fully

anesthetized with isoflurane and a blank or E2 pellet was implanted subcutaneously over the left breast muscle, and the incision was sealed with collodion. All birds were then injected with 0.05cc 0.1mg/ml Eloxiject (Henry Schein Animal Health, Dublin, OH) intraperitoneally for analgesia.

Birds implanted with E2 pellets were injected with 10µl saline into the right breast muscle. Those administered blank pellets were injected with 10µl saline (control group) or 10µl fadrozole (10µg/µl; Sigma-Aldrich, St Louis, MO) (fadrozole group) into the right breast muscle. The initial injection was administered immediately following the implant surgery while birds were anesthetized. Birds were then housed in small cages with one or two other birds of the same treatment group and sex, in a room where auditory and visual contact with the birds in the aviaries was maintained. On each of the following 6 days, each bird was given the same injection of saline or fadrozole into the right breast muscle as initially received. This schedule and dose of fadrozole treatment results in a 2/3 reduction in aromatase activity in the telencephalon of adult male and female zebra finches 24 hours following the last injection [180]. Stimulus Exposure

One day following the final injection of saline or fadrozole, birds were exposed to auditory stimuli in order to evaluate induced ZENK expression. Rhythmic and arrhythmic zebra finch songs were utilized form our previous studies on un-manipulated adult [123] and juvenile [124] zebra finches. All songs used in this experiment were novel to all subjects. Rhythmic stimuli were natural zebra finch songs with no alteration to the timing of the syllables. Arrhythmic stimuli were created by using the same songs utilized as rhythmic stimuli and modifying the duration of the inter syllable intervals in order to disrupt the natural rhythmic structure of the songs. Nine songs of each type were generated, and three subsets of the songs each containing three songs were assembled. Birds were placed, one at a time, into an acoustic isolation chamber (252-Mini Sound Shelter, IAC Acoustics, Bronx, New York, USA), and allowed to habituate for one hour. Each bird was then exposed to one of the subsets of

three songs, with all songs presented at approximately 70dB. One of the three songs was repeated for thirty seconds, followed by thirty seconds of silence. This pattern was repeated for thirty minutes, with the order of the songs randomly determined. Immediately following the presentation of the stimuli, the birds were euthanized by rapid decapitation, and the brains were flash frozen in methyl butane in order to capture peak expression of ZENK mRNA [74]. Blood was collected from the neck following decapitation for analysis of E2 levels. Blood samples were centrifuged for 10 minutes at 10,000rpm at 4°C and plasma was separated and stored at - 80°C until processed for radioimmunoassay. Retention of hormone pellets was confirmed following euthanasia.

Radioimmunoassay

To obtain a general estimate of effectiveness of the hormone manipulations, a single radioimmunoassay was conducted in a manner adapted from Svec and Wade [86]. Parallelism and accurate detection of known quantities of E2 were first demonstrated with recently collected zebra finch plasma (data not shown). Samples were analyzed in three individuals from each combination of conditions (two sexes, three hormone manipulations, and two types of auditory stimuli). To provide an estimate of recovery following extraction, 2000 dpm of 3H-E2 (70 Ci/mmol; NET317250UC; PerkinElmer, Waltham, MA) was incubated at 4°C overnight with 100µl of plasma from control and fadrozole treated birds and 40µl of plasma from E2 treated birds. Steroids were then extracted twice with diethyl ether, and samples were dried under nitrogen. They were resuspended in 5µl of 100% ethanol, then combined with 500µl phosphate buffered saline (PBS) with gelatin and stored overnight at 4°C. A competitive binding assay was completed in duplicate samples, with a serially diluted standard curve (0.98-250 pg E2) in triplicate, by adding an E2 antibody (7010-2650; Bio-Rad AbD Serotec Inc., Raleigh, NC) with 3H-E2 and incubating overnight at 4°C. Six aliquots of a sample containing a known concentration of E2 were used to determine intra-assay precision, and water blanks were added as controls (n = 4). The next day, dextran-coated charcoal (0.025% dextran and 0.25%

charcoal in PBS) was added and centrifuged at 3100rpm for 25min at 4°C in order to remove unbound tracer. The remaining sample was combined with scintillation fluid (UltimaGold; PerkinElmer, Waltham, MA) and analyzed with a scintillation counter (Tri-Carb 2910 TR; PerkinElmer, Waltham, MA). E2 levels were calculated by standardizing samples for individual recovery and the volume assayed and compared to the standard curve. The intra-assay coefficient of variation was 12.7%.

Tissue Processing

Brains were sectioned coronally into 6 series at 20µm and thaw mounted onto SuperFrost Plus slides (Fisher Scientific, Hampton, NH). All series were stored at -80°C until further processing. One series from each animal was stained with thionin to facilitate identification of neuroanatomy.

ZENK mRNA expression in individual cells was visualized by *in situ* hybridization. Bacteria containing a pBlueScript SK+ plasmid with a clone of the zebra finch zenk gene were obtained from the Songbird ESTIMA clone collection [182]. Bacteria were grown overnight on lysogeny broth (LB) agar plates containing 100µg/ml ampicillin. Individual colonies were selected and allowed to grow overnight in LB with 100µg/ml ampicillin. DNA was isolated using the Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI), and the sequence of the insert was confirmed in both directions. Bacteria were then regrown in LB with 100µg/ml ampicillin, and DNA was isolated using the NucleoBond Xtra Maxi kit (Macherey-Nagel, Bethlehem, PA). The DNA was linearized with Sall (T3) and EcoRI (T7) restriction enzymes, and stored at -20°C. Antisense (T3) and sense (T7) probes were transcribed per manufacturer instructions for the Digoxigenin RNA Labeling Kit (Roche Diagnostics, Indianapolis, IN, USA). The probes were purified by filtering through a column made with G50 Sephadex beads and stored at -80°C overnight.

Due to the large number of brain samples, tissue was processed in four runs with all groups represented in every run. One series of slides from each animal was processed using in
situ hybridization for ZENK with antisense probes, and a second series from a minimum of two animals was used in each run for a control to confirm the absence of labeling with sense probes (not shown). Tissue was warmed to room temperature, then fixed in 4% paraformaldehyde for 10 minutes. Slides were rinsed 3x3 minutes in 0.1M PBS, and incubated in 0.25% acetic anhydride in triethanolamine-hydrochloride for 10 minutes. They were then washed 3x5 minutes in PBS and allowed to equilibrate in hybridization buffer for one hour. Slides were incubated at 56°C overnight in 250ng/µl anti-sense or sense probe in hybridization buffer, and the next day rinsed 2x5min in 2X SSC at 60°C. The slides were then incubated in 20µg/ml RNase in 2X SSC at 37°C for 30 minutes, rinsed for 15 minutes in 0.2X SSC at 37°C, then 3x5min in 0.2X SSC at 60°C. The brain sections were allowed to return to room temperature, then rinsed 3x5min in maleic acid buffer with 0.1% Tween-20 (MABT). The slides were incubated in 0.9% H₂O₂ in MABT for 30 min and rinsed 3x5min in MABT. They were incubated in 5% normal sheep serum in MABT for 30 min, then rinsed 3x5min in MABT. Next, the slides were incubated in 0.5µl/ml anti-digoxigenin-AP Fab fragments (Roche Diagnostics, Indianapolis, IN) in MABT for 2 hours, then rinsed 2x3min in MABT. Slides were rinsed 3x5min in detection buffer to equilibrate. The color reaction was performed by incubating slides in 4.5µl/ml NBT and 3.5µl/ml BCIP (Roche Diagnostics) in detection buffer for 1 hour 55min. Slides were rinsed 3x5min in TE buffer to stop the color reaction, then dehydrated and coverslipped with VectaMount (Vector Laboratories Inc., Burlingame, CA, USA).

Data Analysis

A few animals were excluded from analysis of a particular brain region due to damage to individual tissue sections. Final sample sizes are indicated in Table 4. Separate mixed model ANOVAs were conducted for each brain region with hemisphere as a within subjects factor and sex, hormone manipulation, and stimulus type (rhythmic or arrhythmic) as between subjects factors. A main effect of hormone in CMM was followed with a *post hoc* Scheffe test to determine which pairs of hormone conditions differed. Two-way and three-way interactions

detected within NCM were each probed with subsequent ANOVAs within groups (see Results for details). Bonferroni corrections were used to adjust for multiple comparisons, with adjusted α -levels indicated with each result. All statistics were calculated using SPSS (IBM, Armonk, NY).

Results

Hormone manipulation

Two samples were eliminated from analysis due to technical mistakes. Five samples, two from fadrozole-treated and three from the control birds, were below the limit of detectability for this assay. To be conservative they were assigned the lowest detectable value for the purpose of statistical analysis. A significant effect of hormone treatment on circulating E2 levels was detected ($F_{2,31}$ =150.10, p<0.001). A post hoc Scheffe test indicated a greater concentration of the hormone in the E2-treated (M=3.53ng/ml, SEM=0.26, n=12) compared to both the control (M=0.15ng/ml, SEM=0.01, n=11) and fadrozole-treated (M=0.16ng/ml, SEM=0.01, n=11) groups (both p<0.05). However, the values were equivalent in the control and fadrozole-treated groups (p>0.05).

CMM

A significant main effect of hemisphere was detected, with a greater density of ZENK expressing cells in the left hemisphere than the right ($F_{1,88}$ =35.917, p<0.001; Figure 11). A main effect of sex was detected ($F_{1,88}$ =8.557, p=0.004; Figure 11), such that females had a greater density of ZENK expressing cells than males. The hormone manipulation also produced a significant effect on ZENK expression ($F_{2,88}$ =3.468, p=0.036; Figure 11). On average, the density of ZENK+ cells was highest in the control birds compared to those with hormone manipulations, however *post hoc* comparisons indicated no significant differences between pairs of these groups. No main effect of rhythm stimulus type was detected ($F_{1,88}$ =1.308,

p=0.256), and no significant interactions were detected between any of the variables (all *F*<1.338, *p*>0.267). Data for individual groups are presented in Table 4.

Figure 11. Density of cells expressing ZENK in CMM. Panel A depicts this measure across sexes and hormone conditions (mean ± SEM). Data are collapsed across stimulus type (rhythmic and arrhythmic), as no significant effects of this variable were detected. A main effect of hemisphere, with greater density of ZENK expression on the left, is indicated by *. A main effect of sex (female > male) is indicated by different letters within the bars. The photographs in panel B depict representative examples of ZENK expression in birds exposed to arrhythmic song in the left and right hemisphere of a female, and the left and right hemisphere of a male.





Right



Male









Table 4. ZENK+ cells/mm² means (standard error) for the caudomedial nidopallium (NCM), caudomedial mesopallium (CMM), and nucleus taeniae (Tn). The sample size is indicated on the second row of each cell.

			Fadro	ozole	Cont	trol	Estra	diol
		1	Male	Female	Male	Female	Male	Female
		Rhythmic	433.5 (60.6) 7	431.9 (32.4) 9	486.0 (59.2) 7	520.0 (48.1) 9	382.9 (39.0) 9	502.2 (43.4) 9
	KIGNT	Arrhythmic	344.5 (61.4) 7	430.3 (33.5) 9	437.9 (41.6) 9	552.3 (49.2) 9	334.0 (59.8) 7	450.4 (53.5) 9
CIMIM	-	Rhythmic	486.7 (59.7) 7	519.1 (53.4) 9	564.4 (90.8) 7	613.3 (63.8) 9	416.7 (46.6) 9	559.9 (60.5) 9
	Геп	Arrhythmic	393.6 (71.0) 7	588.2 (67.3) 9	505.5 (60.0) 9	570.3 (38.3) 9	393.6 (74.7) 7	500.5 (58.9) 9
		Rhythmic	285.7 (56.4) 7	315.1 (61.0) 9	499.7 (79.9) 7	259.0 (31.6) 9	321.3 (69.9) 9	264.4 (38.2) 9
(KIgnt	Arrhythmic	256.7 (62.6) 7	306.8 (54.5) 9	410.6 (66.4) 9	217.9 (37.9) 9	287.4 (101.8) 7	341.4 (33.8) 9
	-	Rhythmic	302.6 (45.1) 7	357.5 (65.5) 9	431.0 (52.8) 7	259.3 (25.6) 9	394.5 (102.9) 9	339.3 (85.3) 9
	Lett	Arrhythmic	307.5 (71.6) 7	279.1 (52.0) 9	532.2 (120.2) 9	266.2 (42.1) 9	420.3 (92.34) 7	352.7 (41.7) 9
	+4 -4 -2 	Rhythmic	248.8 (32.2) 7	348.7 (47.4) 9	416.3 (80.2) 7	315.1 (43.3) 9	199.2 (41.4) 9	328.5 (62.6) 9
s F	KIBIIL	Arrhythmic	225.4 (52.2) 7	330.5 (54.4) 9	280.7 (63.1) 9	215.5 (32.5) 9	192.1 (62.6) 7	300.8 (57.6) 9
=	- -	Rhythmic	258.6 (32.1) 7	394.6 (49.7) 9	474.1 (70.7) 7	476.1 (43.1) 9	289.3 (53.1) 9	364.0 (70.8) 9
	reit	Arrhythmic	378.1 (98.0) 7	349.6 (75.6) 9	347.7 (57.7) 9	332.4 (54.1) 9	264.8 (52.3) 7	369.7 (59.4) 9

<u>NCM</u>

As seen in CMM, a significant main effect of hemisphere was detected with a greater density of ZENK expressing cells on the left compared to the right ($F_{1,88}$ =8.586, p=0.004; Figure 12). An increase in these cells was also found in males compared to females ($F_{1,88}$ =4.253, p=0.042; Figure 12). No main effects of stimulus rhythmicity ($F_{1,88}$ =0.014, p=0.908) or hormone manipulation ($F_{2,88}$ =0.886, p=0.416) were detected.

A significant interaction existed between sex and hormone type ($F_{2,88}$ =4.196, p=0.018; Figure 13). This result was probed by conducting separate one-way ANOVAs within each sex with hormone condition as a factor, as well as within each hormone condition with sex as a factor. No effect of hormone type was detected in males or females ($F_{2,43}$ =2.915, p=0.065; $F_{2,51}$ =1.541, p=0.224 respectively; α =0.025). Within control birds, a greater density of ZENK expressing cells was present in males compared to females ($F_{1,32}$ =13.902, p=0.001; α =0.017), whereas no effect of sex was detected the fadrozole- ($F_{1,30}$ =0.213, p=0.648; α =0.017) or E2treated ($F_{1,32}$ =0.216, p=0.645; α =0.017) animals.

In addition, a significant three-way interaction was detected among hemisphere, sex and rhythm stimulus type ($F_{1,88}$ =5.164, p=0.025; Figure 12). This result was further investigated with multiple two-way ANOVAs, followed by appropriate pairwise comparisons. The interaction was clearly driven by an increased density of cells expressing ZENK mRNA in the left compared to the right hemisphere of arrhythmic song exposed males ($F_{1,22}$ =8.509, p=0.008, α =0.0125; Figure 12).

None of the other possible interactions among hemisphere, sex, rhythm type, and treatment were statistically significant (all *F*<2.578, *p*>0.081). Data for the individual groups are presented in Table 4.

Figure 12. ZENK expressing cells in NCM. Panel A depicts the density of cells containing ZENK mRNA across the sexes and auditory stimuli (mean \pm SEM). A main effect of hemisphere, with greater expression on the left, is indicated by *. Different letters within the bars indicate a main effect of sex, with greater ZENK expression in males compared to females. A three way interaction was detected, greater ZENK density was seen on the left than the right in arrhythmic song exposed males, indicated by Δ . Panel B shows pictures of representative samples of ZENK expression in control males and females exposed to rhythmic song.







Figure 13. ZENK expression in the NCM of males and females across hormone manipulations.

Panel A depicts the density of cells (mean ± SEM) across the three treatment groups. An interaction was detected; expression was greater in males than females only in the control animals, indicated by *. Panel B shows representative images from a control female and male.



<u>Tn</u>

Similar to both auditory regions, a significant main effect of hemisphere was detected, with a greater density of ZENK expressing cells in the left compared to the right hemisphere ($F_{1,88}$ =18.936, p<0.001). No main effect of sex ($F_{1,88}$ =2.545, p=0.114), rhythmic stimulus type ($F_{1,88}$ =2.324, p=0.131) or hormone manipulation ($F_{2,88}$ =1.963, p=0.147) was detected.

Significant interactions were also not detected among any combination of hemisphere, sex, rhythm type, and hormone (all *F*<2.606, p>0.079). Data are presented in Table 4.

Discussion

Summary of Specific Effects

Across NCM, CMM and Tn, a greater density of cells expressing ZENK mRNA was detected in the left hemisphere. Sex differences in ZENK expression were detected in both auditory regions, although in opposite directions, with a higher density of cells in males in NCM and in females in CMM. An effect of hormone manipulation was also seen in CMM, and while no pairwise comparisons were statistically significant, the pattern suggested greater ZENK expression in the control birds compared to both the E2- and fadrozole-treated groups.

Statistically significant interactions were detected just in NCM. In the control group only, the density of cells expressing ZENK mRNA was greater in males than females. ZENK expressing cells were also increased in the left compared to the right hemisphere in males exposed to arrhythmic, but not rhythmic, song. Thus, these groups contributed substantially to the main effect of hemisphere. In the present study, effects of rhythmicity of the song stimulus were limited, but may have been reduced by particular methodological differences from our earlier studies [123, 124]. Individual effects are interpreted in more detail below.

Lateralization

The finding in the present study of a higher density of cells expressing ZENK mRNA in the left hemisphere adds to a complex literature on lateralization of auditory perception. In humans, left lateralization of linguistic functions appears consistent across production and perception (reviewed in [29]). These results parallel the left lateralization following perception of conspecific vocalizations in the present study. However, the literature in songbirds is less consistent than that from humans. Similar to the present experiment, increased ZENK expression was detected in the left NCM of juvenile zebra finches exposed to novel conspecific

song [183]. However, the same study did not find lateral differences in adults. Our previous work on juvenile zebra finches, using the same auditory stimuli as the current study [124], did not initially assess hemisphere as a factor, but further analysis of the data reveals no significant differences in the density of cells expressing ZENK protein between the left and right sides of the brain. Electrophysiological studies have also provided mixed results. Increased response strength in the left hemisphere has been detected among fast-learning birds trained in a GO/NoGO paradigm [184]. Zebra finches exposed to four or nine days of a heterospecific acoustic environment also showed left lateralization of electrophysiological responses in NCM [185], but those housed under typical conditions with conspecific song exposure showed right lateralization of NCM activity [185, 186]. An fMRI study indicated greater differences in response strength between bird's own song, novel conspecific song, tutor song and tones in the right hemisphere of the zebra finch brain [84]. Given the inconsistencies that can be detected across different measures of neural activity, and the fact that it is possible for neurons to fire without inducing ZENK expression (reviewed in [91]), further work is necessary to draw conclusions about the functional significance of lateral differences in gene expression detected in the present study.

<u>CMM</u>

The sex difference in CMM, with greater ZENK expression in females compared to males, parallels results from our previous study with adult zebra finches without hormone manipulations [123]. The quality of songs is an indicator to females of the fitness of males [187]. Thus, the consistent increase in activity in females compared to males in CMM may relate to females' use of CMM for evaluation of songs of potential mates, a function typically not employed by males.

The trend for increased ZENK expression in control animals compared to those treated with either E2 or fadrozole suggests that an optimal level of the hormone may exist. This pattern of results is consistent with a study that found a trend in NCM for a reduced

electrophysiological response to novel conspecific song in fadrozole- and E2-treated birds compared to saline-treated controls [188]. Together these results suggest that too much or too little available E2 within the auditory forebrain may inhibit optimal neural responses to auditory stimuli. Fadrozole can reduce both the aromatase activity within the zebra finch brain [180] and the total amount of E2 present in the telencephalon [189]. While our assay did not detect differences in plasma E2 levels between the control and fadrozole treated birds, those receiving the estrogen synthesis inhibitor likely experienced less E2 availability in the auditory brain due to decreased local synthesis as well as a possible reduction in the E2 released within NCM during song exposure [108, 122].

<u>NCM</u>

In contrast to the effect seen in CMM, a greater density of cells expressing ZENK mRNA was seen in males compared to females in NCM. While this effect was not detected in our previous study [123], a similar pattern of activity was detected in the NCM of zebra finches using electrophysiology, with males showing an enhanced response to novel conspecific songs compared to females [188]. The interaction between hormone manipulation and sex in the present study indicates that this sex difference is limited to the control animals. Similar to the present data from CMM, this result suggests that there may be an optimal level of circulating E2, and an increase or decrease can diminish sex differences in auditory processing. In addition, the opposite directions of sex differences between NCM and CMM may indicate that these areas are functionally specialized. Specifically, NCM may be more involved in processing by males for nest site defense, whereas CMM may aid in processing of song for value as a potential mate.

The interaction between sex, hemisphere, and rhythmicity of the song stimuli in NCM could present a challenge for interpretation. A significant increase in ZENK expression in the left relative to the right hemisphere was detected only in males exposed to arrhythmic song; it is difficult to speculate on potential reasons for such a specific effect. However, a trend for a

greater density of ZENK+ cells in the left hemisphere existed in all other combinations of sex and rhythm type. Thus, while data from these males was largely responsible for the main effect of lateralization in NCM, it is unlikely that this interaction represents a functional difference across groups.

Tn

The lack of effect of stimulus rhythmicity detected in Tn contrasts with the results previously seen in adults that did not receive hormone manipulations [123], but both studies are consistent in finding no sex differences in ZENK expression in Tn. It would be parsimonious to suggest that the hormone manipulations in the present study eliminated the increased response to arrhythmic song [123], however the control birds also responded similarly to the two types of auditory stimuli (Table 4). Thus, other differences from our previous study likely influenced the results.

Methodological Issues

The lack of effects due to song stimulus type differs from our previous finding of an increased density of ZENK+ cells in response to arrhythmic compared to rhythmic song across all three brain areas investigated [123]. Several methodological aspects may relate to the differences between studies. For example, birds in the present study were housed with only one or two conspecifics, whereas they were kept in large group aviaries in our earlier work. It is possible that the current conditions were more stressful. Keeping zebra finches in isolation while maintaining auditory and visual contact with conspecifics can cause changes in ZENK expression in the social behavior network, which includes Tn [190]. The experience of undergoing anesthesia, a hormone implant, and a week of daily injections likely also induced some stress compared to our earlier study on adults, which could have limited ZENK responses both directly by changing the neurochemical environment and indirectly by reducing the birds' attention to conspecific vocalizations.

Perhaps the most important difference between our studies, however, involved the quantification of ZENK protein in the earlier work [123] versus mRNA in the present study. This change was made because the antibody used in the previous study was no longer available. While levels of mRNA and protein expression are often correlated, they are not always consistent, and the degree of correlation can vary widely between genes and tissue types [191, 192]. It is possible that the pattern of ZENK responses to the stimuli presented in this study differs between mRNA and protein, contributing to the variation of the present results from the expected pattern based on previous research.

Our results also differ from studies indicating E2's capacity to improve auditory discrimination to types of stimuli not used in the present experiment, such as conspecific vs. heterospecific song, tones, reverse song, or white noise [108-111, 193]. It is unclear whether E2 can also modulate responses to auditory rhythms, as the anticipated difference between our stimulus types was not detected. Further work is needed to resolve this issue.

NCM is a large, heterogeneous brain region. In white-throated sparrows, E2 enhances the ZENK response to song compared to tones in the rostral and medial portions of NCM, but not in other regions of the nucleus [193]. The white-throated sparrow brains were sectioned in the sagittal plane [193], whereas the brains in the present study were sectioned coronally in order to maintain consistency with our earlier work [123, 124]. While the area of NCM analyzed here is likely in the rostral region described in the sparrow study, it is difficult to make exact comparisons across different planes of section. If zebra finches are similar in the regional specificity of E2 sensitivity within NCM, it is possible that areas of NCM other than what was sampled in the present study would show greater differences in ZENK expression between hormone groups.

It is also possible that the hormone manipulations did not provide sufficiently large differences in brain E2 availability from the control group. However, we think this is unlikely. While our assay was not sensitive enough to detect a significant reduction in circulating E2 in

fadrozole compared to the control birds, the same manipulation substantially reduces aromatase in the forebrain as a whole [180], where NCM, CMM and Tn are located. The pattern of ZENK expression across manipulations also indicates that fadrozole was effective in the brain, as the sex difference detected in NCM in control animals was eliminated by this drug.

A final methodological consideration is the duration of the hormone manipulations. E2 is rapidly synthesized in the NCM of zebra finches in response to conspecific song [108, 122]. In addition, the effects of E2 on auditory responses can appear rapidly and are likely mediated through the membrane bound receptor GPER1 (previously known as GPR30) [119]. Continuous exposure to E2 for one week may have overwhelmed these rapid E2 responses, washing out any modulation of auditory response that increased E2 may facilitate. We chose to use a longer term E2 modulation because it provided a minimally invasive way to influence E2 levels that had the possibility of impacting modulation through multiple E2 receptor types [180]. Future studies using infusion of fadrozole or E2 into NCM could allow for assessment of rapid E2 effects on rhythm perception.

<u>Conclusions</u>

Overall the results of the present study suggest a strong left lateralization of neural activity in response to conspecific songs across brain regions, regardless of available E2. This effect parallels the lateralization of language seen in humans. In addition, the data suggest the possibility of an optimal level of E2 at which these neural responses are strongest. Sex differences were also opposite between auditory regions, with greater activity in NCM in males and in CMM in females. Further studies are needed to clarify potential relationships between E2 and rhythm perception, as well as the functional significance of sex differences and lateral differences in gene expression.

CONCLUSION

Rhythm is a central component of both birdsong and human language. A large variety of human disorders involve deficits in rhythm and timing perception, including speech and language, developmental and neurodegenerative disorders. The neural basis of rhythm perception and its relation to these deficits is still poorly understood. Developing the zebra finch as a model will allow investigations of the neural underpinnings of rhythm perception, as well as hormonal influences on this process, in a vocal learner in ways not possible in humans.

The experiments in this dissertation set out to examine the influence of auditory rhythms on neural activity in multiple nuclei in the auditory and song systems and one nucleus in the social behavior network. In addition, I examined the development of neural responses to rhythm in order begin to determine whether increased responses to arrhythmic song detected in the adult brain are present prior to song learning, or acquired during particular stages. I also assessed whether adult neural responses to rhythmicity are modulated by E2.

Results showed that arrhythmic song induced more ZENK expression than rhythmic song in two regions of the auditory cortex (the caudomedial nidopallium, NCM, and caudomedial mesopallium, CMM), as well as in the avian homolog of the amygdala (nucleus taeniae, Tn). The increased ZENK expression in NCM and CMM of birds exposed to arrhythmic compared to rhythmic song suggests that these modified songs were similar enough to natural zebra finch songs to be processed as conspecific song, but birds may have detected errors relative to the expected song pattern. Greater ZENK expression in females compared to males in CMM indicates that this region may be involved in assessment of mate songs in females, as a comparison to the template that is believed to be stored there as well as in NCM (reviewed in [194]), a function that is not typical of male zebra finches as females do not sing. In birds, Tn activity is associated with pair bonding and mating activities [129, 141, 142], and in its human homolog, the amygdala, increased activity occurs with exposure to aversive auditory stimuli [146, 147]. Taken together, these ideas suggest that increased activity in Tn in response to

arrhythmic song may indicate a negative response to poor quality song. This idea may be particularly relevant for females who use song in mate selection [36, 187], but the lack of a sex difference in Tn suggests that males respond to the undesirable characteristic of the stimulus in a similar way.

In contrast to the rhythmic differences seen in NCM, CMM, and Tn, no effects of rhythm were present in the anterior forebrain pathway (involved in song learning and plasticity, [61]), or the vocal motor pathway (critical for song production, [36]) of the song system. In the anterior forebrain pathway, greater ZENK expression was present in the medial compared to the lateral striatum, with this difference significantly enhanced in males compared to females. A major factor in this sex difference is that the song control region Area X occupies the lateral striatum in males, but is not present in females. A clear absence of ZENK expression was seen within Area X, while sparse labeling was seen in the lateral striatum in females. This result suggests that the medial striatum is involved in auditory processing, unlike Area X or the lateral striatum in females, but is not directly involved in rhythmic discrimination. Unlike the striatum, little or no ZENK was detected in LMAN across both sexes and stimulus types. In the vocal motor pathway, little or no ZENK expression was detected across sex and stimulus type within the premotor nucleus HVC. Due to their uniform lack of response, both LMAN and HVC appear not to be involved in rhythmic processing of novel conspecific songs, although it is possible that HVC could discriminate rhythm in other stimulus types, such as a bird's own song or a tutor's song.

In assessing the development of differential responses to rhythmic and arrhythmic stimuli, I found that prior to template formation (post-hatching day 15, d15), NCM shows increased ZENK expression in response to rhythmic compared to arrhythmic song, indicating an unlearned preference for more natural conspecific vocalizations. In contrast to NCM, where no rhythmic differences were present at d25 or d45, an adult-like pattern was detected in CMM during template formation (d25), with greater ZENK expression in birds that heard arrhythmic

song, but not during sensorimotor integration (d45) when no rhythm effects were detected. One possible explanation is that birds are focused around d25 on accurately perceiving external stimuli for creation of the highest fidelity match and this focus on auditory perception is important again in adulthood when the quality of a song is important for mate attraction and nest site defense. In contrast, during sensorimotor integration around d45, male zebra finches are more focused primarily on the motor task of matching their own vocalizations to a preexisting template. The zebra finch brain may have a greater capacity to discriminate auditory stimuli during times when primary attention toward auditory signals is ecologically relevant, and both sexes may show a reduced capacity to discriminate rhythms at ages important for sensorimotor integration in males. A lack of effects found Tn suggest that ZENK responses to the rhythmicity of song in this region develop between post-hatching day 45 and maturity.

E2's effect on rhythmic processing remains unclear, though some effects of E2 were detected. There appeared to be an optimal level of E2 availability in the brain, with the greatest level of ZENK induction in CMM seen in birds with unmanipulated levels of E2, and reduced responses in groups with elevated or suppressed E2. Within birds in the E2 manipulation study, opposite sex differences were found in NCM and CMM with greater responses in females in CMM and males in NCM. This sex difference could suggest that NCM is more involved in conspecific processing by males, whereas CMM is more involved in females. The suggestion of an optimal level of E2 availability is strengthened by the sex difference in NCM being driven by the control animals, with no sex differences seen in the fadrozole or E2 treated groups. In contrast with my earlier findings, no rhythm differences were detected across hormone treatments in Chapter 3. The lack of rhythm effects in all groups, including the control birds, in the study in which E2 availability was manipulated in adults limits the conclusions that can be drawn about whether E2 is involved in rhythm processing. Several differences between this study and the initial adult rhythm perception study (Chapter 1) could have contributed to the differences in detection of rhythm effects. For example, in Chapter 3, I measured ZENK mRNA

whereas the initial study in adults (Chapter 1) I assessed protein, and the manipulations likely induced stress compared to the original study. While the expected effects of stimulus rhythmicity were not seen in the experiment involving E2 manipulation, strong left lateralization of ZENK induction by song was detected in contrast to the unmanipulated adults and juveniles. This effect was present across brain regions investigated, stimulus types, and hormone treatments, paralleling the left lateralization of language seen in humans. The literature addressing lateralization of neural responses to auditory stimuli in zebra finches is inconsistent, with different findings under varied experimental conditions. As with the differences in detection of rhythm effects between studies in this dissertation, the detection of lateral differences in the E2 manipulated birds, but not others could be due to the measurement of mRNA rather than protein, or the stress experienced as a result of the hormone manipulations. Further studies are needed to elucidate the role of E2 both rapidly and long term, on rhythm perception.

The neural discrimination of rhythmicity detected was variable across studies in all brain regions analyzed. In CMM, increased ZENK expression in response to arrhythmic song was detected at d25 and also in adulthood suggesting that rhythm discrimination may be enhanced during life stages when birds are most focused on perception of external auditory stimuli. In the E2 manipulation study, no effect of rhythm was present in CMM. This difference could have been due to differences between mRNA and protein expression, but could also be an indication that the stress induced by the manipulations in this protocol reduced the receptivity for and attention to external auditory stimuli, thus decreasing rhythmic discrimination. In NCM, increased ZENK expression was detected in response to rhythmic song at d15 and to arrhythmic song in adulthood. This change in pattern between the early juvenile period prior to template formation and adulthood suggests that adult rhythm discrimination may have a learned component, with increased responses to arrhythmic song indicating detection of errors relative to the learned template. In Tn, rhythm differences were detected only in the initial adult study, but not in the juveniles or E2 treated birds. The perception of the quality of social signals in Tn

likely develops between d45 and adulthood, during the late juvenile period. In all regions analyzed, the lack of rhythm effects in the E2 manipulation study in comparison to unmanipulated birds could be due to either differences between mRNA and protein expression, or the effects of stress minimizing responsiveness and discrimination.

Across studies in adult zebra finches, greater density of ZENK expressing cells was present in females compared to males in CMM, but no sex differences were detected in juveniles. Together this suggests that CMM plays a larger role in assessing conspecific signals in females, potentially for the purpose of mate quality assessment, and that this sex difference develops in the late juvenile period. In NCM a sex difference was found only in the control treated birds in the E2 manipulation study with greater ZENK density in males compared to females, but not in juveniles or unmanipulated adults. Increased response magnitude in NCM in males compared to females in response to novel conspecific song has previously been detected in zebra finches [188]. Yet other studies have measured no differences in response between the sexes [195]. Thus NCM may have some specialized function that necessitates greater activity in males, but it is unclear what experimental factors influence the detection of sex differences in this region. No sex differences were seen in Tn in any of the experiments in this dissertation. Given the role of Tn in pair bonding and mating behavior, it would have been unsurprising to find increased activity in females in response to male song, but this was not the case. The data suggest that Tn is involved in evaluating conspecific song, and potentially the aversive nature of disrupted song, in both sexes, independent of any assessment for mate quality in males.

The research in this dissertation represents the first step in establishing the zebra finch as a model for human rhythm perception and disorders involving rhythm deficits, particularly stuttering. These experiments provide an initial understanding of how the adult zebra finch brain processes rhythm, how rhythm processing develops in juveniles, and the effects of E2 manipulations on the adult brain. The foundation laid by these experiments provides the

opportunity for further study into the mechanisms of rhythm perception in a way that would not be possible in humans. Ultimately it would be informative to conduct parallel studies to analyze both behavioral and neural responses to rhythm in both humans and zebra finches. An initial study that would be of value to allow additional mechanistic research would be to establish behavioral discrimination or a behavioral preference between rhythmic and arrhythmic song in zebra finches. Multiple test designs could be utilized for this purpose. A preference test where the two types of songs are played at opposite ends of a long cage and the amount of time the bird spends in proximity to each speaker is evaluated could provide information regarding the birds' preference regarding rhythmicity. Another way to assess preference would be to link playback of each song type to pecking a particular key or jumping on a particular perch, and assessing which stimulus is played more. An additional methodology that may allow behavioral assessment of rhythm discrimination would be a go/no-go paradigm, where birds are trained to take a particular action, such as pecking a key, when exposed to one type of stimulus, and withhold that response when exposed to the other type of stimulus. Once a consistent behavioral discrimination was established using one of these, or a similar methodology, further studies could assess how various manipulations within the brain could influence the behavior patterns.

Among the many mechanistic studies that could be conducted following the work of this dissertation would be the inactivation of the regions that show differential responses to rhythm, NCM, CMM, or Tn, and then assessment of the impact on behavioral responses, or on ZENK expression in the other areas not inactivated. This could be done permanently through the use of chemical or electrolytic lesions, or transiently through the use local cooling or infusion of an inhibitor such as tetrodotoxin that prevents the firing of action potentials. It would also be informative to assess the phenotype of the cells expressing ZENK following exposure to rhythmic and arrhythmic song. Double label immunohistochemistry or in situ hybridization for ZENK and markers of various neurotransmitter systems could identify the types of cells

responding to the stimuli. One cellular phenotype known to be present in NCM is GABAergic cells. It would be informative to assess the effect of a local infusion of a GABA antagonist into NCM prior to a behavior test to determine if inhibitory processes influence discrimination.

Further studies assessing the influence of E2 on rhythm discrimination would also be valuable. Local infusions of E2 or fadrozole into NCM, CMM, or Tn followed by behavioral assessment, or assessment of ZENK expression could illuminate the influence of fast acting estrogen receptors in rhythm perception. It would also be valuable to conduct microdialysis in NCM, to determine whether there are differences in induction of E2 synthesis between exposure to rhythmic and arrhythmic song.

This body of work has implications for the growing field of rhythm and timing perception in song birds, as well as more broadly for vocal learning species and human rhythm perception. Improving our understanding of rhythm perception in songbirds can provide insight and basis for future research in healthy humans, as well as those with disorders involving deficits in rhythm perception. The studies contained in this dissertation demonstrated rhythmic discrimination in neural responses in NCM and CMM which parallel the human auditory association cortex, and in Tn, which shares homology with the human amygdala [129]. These results suggest that the auditory association cortex and amygdala would be valuable brain regions to assess for their involvement in human rhythm perception in general, as well as for any deficits in those with disorders that involve deficits in rhythmic processing. Few studies have assessed rhythmic processing in these areas. In parallel to the increased activity detected in NCM and CMM in response to arrhythmic song in the initial adult study, arrhythmic tone sequences induce greater activity in the secondary auditory cortex in humans [135]. Rhythmic drumming produced by rhesus macaques induces activity in their amygdala [196]. Further studies comparing the amygdala's response to rhythmic and arrhythmic sequences in monkeys, or assessing its response to rhythmicity in humans have not been conducted. More studies are warranted to assess whether these brain regions are implicated in any rhythm processing deficits.

The second study in this dissertation indicated that aspects of rhythmic processing may be learned. This idea suggests that rhythmic training may improve processing and aid those with speech and language disorders involving rhythm deficits. Continued study to improve our understanding of E2's impact on rhythmic processing could aid in our understanding of sex differences in prevalence of disorders involving rhythm deficits, such as stuttering which is more common in males than females [13]. The adult studies in this dissertation consistently found greater activity in CMM in females compared to males; perhaps if a similar pattern is present in humans, greater auditory response to rhythmicity in auditory regions of the female brain could contribute to the lower rates of stuttering. Further study of E2 and rhythmic processing could also shed light on why rates of spontaneous recovery among children who stutter are much greater in females [13]. Spontaneous recovery typically occurs by puberty [13], thus it is possible that higher rates of spontaneous recovery in females are due to increasing levels of E2 facilitating improved rhythmic processing as they approach puberty. The lower rates of stuttering in adults also parallel the greater rhythm discrimination detected in adult compared to juvenile zebra finches. Further research is necessary to improve our understanding of continued development of rhythm perception in the late juvenile period, E2 influences on rhythm perception, and factors that may disrupt the capacity for neural discrimination of rhythm in zebra finches.

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