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Testing the Template Hypothesis of Vocal Learning in Songbirds.

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Graduate Program in Neuroscience

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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Testing the Template Hypothesis of Vocal Learning in Songbirds.

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by

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Graduate Program in Neuroscience

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

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Abstract

The auditory forebrain regions NCM and CMM of songbirds are associated with perception and complex auditory processing. Expression of the immediate-early gene ZENK varies in response to different sounds. Two hypotheses are proposed for this. First, ZENK may reflect access to a representation of song memories. Second, ZENK may reflect attention. I tested these hypotheses by measuring ZENK in response to tutored heterospecific or isolate songs compared to non-tutored wild-type song. Young zebra finch females were exposed to different tutoring conditions and later exposed to different playbacks, and the expression of ZENK in CMM and NCM measured. ZENK responses varied across playback stimuli in some brain regions, but did not interact with tutoring conditions. These results do not support the hypothesis that ZENK activation reflects auditory memories.

Keywords

Vocal learning, forebrain auditory areas, birdsong and speech, birdsong and speech acquisition, template hypothesis, immediate early genes, innate template.
Co-Authorship Statement

Publications derived from this research will be co-authored by Scott MacDougall-Shackleton.
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Chapter 1

1 Introduction

To speak a language is a complex and exclusive human faculty. However, in order to perform this unique trait humans require a vocal learning ability, the capacity to imitate or modify complex vocal sounds. Unlike language, vocal learning is not unique to humans, and though it is still a rare trait in mammals it is widely spread among birds. Vocal learning involves the imitation of species-specific communication sounds leading to speech learning in humans and to song learning in birds (Brainard & Doupe, 2002; Jarvis, 2007; MacDougall-Shackleton, 2009; Bolhuis, Okanoya, & Scharff, 2010). Songbirds have thus become a widespread behavioral and neural model that allows us to research vocal learning as a perceptual and motor ability (Brainard & Doupe, 2002).

The neural bases of vocal learning has been studied with respect to the underlying structures and circuits that support the process of learning and production of the motor programs required to develop an effective communication signal; and its interaction with the system that allows the detection, discrimination, identification, selection, recognition and memorization of the sounds that are relevant to the vocal learning process (Brainard & Doupe, 2002; Catchpole & Slater, 2008).

Birdsongs as communication signals seem to have an adaptive role that influences the behavior of the receivers. In order for this signal to be effective, a predictable connection between production and perception of natural complex stimuli (song) in a behavioral context appears to be required. Therefore, songs need to be processed to establish their relevance in a specific context and, presumably, based on this process the receiver will generate a proper response. This response is usually related to cognitive process such as, memory, learning and attentional mechanisms (Knudsen & Gentner, 2010).

Song in songbirds seems to be one of the most relevant traits in competition for mates, resources, and advertising. Being a sexually selected trait, singing is usually dimorphic so males tend to be the ones that exhibit the behavior, and females are the receivers. In most species of songbirds females have the task of selecting the male, and male’s song is one
of the key traits assessed (Catchpole & Slater, 2008). Therefore, females need to process the song and generate the proper response. This response can be measured at behavioral and neural levels. To explore the neural processing of song, different experimental techniques such as: awake behaving animal responses, single unit recording, autoradiography, functional magnetic imagining fMRI and gene expression have been used (Theunissen & Shaevitz, 2006).

In this thesis I explore the influence of early tutoring conditions on the adult neural responses (specifically using the gene expression technique) of female zebra finches (the receiver) to different stimuli in two auditory forebrain areas that have been proposed to play a fundamental role in the perceptual processing of song. The function of these regions is still a matter of debate. On one hand, these brain areas seem to be functionally associated with the sound characteristics relevant to the species – specific songs (nature of the stimuli), and, on the other hand, with the memory of the song formed during the song acquisition process (familiarity of the stimuli). In this introduction I will initially present an overview of vocal learning and similarities between humans and songbirds, then I will focus on birdsong (function, acquisition, and the neural structures involved). Finally, I will focus on song processing in female songbirds, specifically in two auditory areas, and the possible functions of these structures based on the outcomes from studies using gene expression.

1.1 Vocal learning - Speech and Birdsongs.

Humans and birds are some of the few species that possess the ability to imitate complex vocal sounds. As a result, songbirds have been extensively studied with respect to this trait because of the similarities that they exhibit in the acquisition of birdsong as compared to speech acquisition in humans. Songbirds require auditory experience in the process of song acquisition. In fact young birds learn their songs by imitating usually the song of conspecific models that they hear. This point is further discus in section 1.2

In both humans and songbirds, it seems that a combination of a predisposition to learn and experience are required for vocal development (Brainard & Doupe, 2002). Children seem to be born equipped with special capacities and knowledge of language and speech
in advance of experience. Indeed, infants are able to recognize a broad repertoire of phonemes from different languages, even if they are not included in the subset of phonemes used by their caregiver’s language. However, this ability is progressively narrowed through development during interaction with speakers from a specific language or dialect (White, 2003; Guasti, 2004; Doupe & Kuhl, 2008). In a comparable way, young songbirds seem to begin life with foreknowledge about their species’ song. In fact, they seem to have a selective predisposition to learn the sounds from their own species even if they are able to produce sounds from a different species (Catchpole & Slater, 2008; Doupe & Kuhl, 2008). This is the case in male swamp sparrows (Melospiza georgiana; Marler & Peters, 1977). Male swamp sparrows were exposed early in life to tape recordings that contained syllables of their own species and to the syllables of sympatric song sparrows (Melospiza melodia). Interestingly, the young swamp sparrows, in their final song, included only the elements from the swamp sparrow songs. This preference could reflect constraints that emerge even with little influence of the environment and they seem to limit the bird’s attention to the species relevant stimuli (Marler & Peters, 1977). The fact that they favored their conspecific song, compared to a heterospecific song, indicates that songbirds may have some bias restrictions that guide the song acquisition process and include the acoustic features that the species-typical song should contain. Consequently, birds memorize songs that correspond to their innate template (Searcy, Marler, & Peters, 1985; Marler, 1997; Bolhuis & Gahr, 2006; Fehér, Wang, Saar, Partha, & Tchernichovski, 2009, Bolhuis, Okanoya, & Scharff, 2010).

However, it also has been proposed that this bias towards their own species song could arise, in part, from the fact that the song exposure in most research was done using tape recordings instead of live tutors, and thus social interactions between the song tutor and tutee were eliminated (Catchpole & Slater, 2008).

It is also suggested that during the song learning process songbirds are able to memorize a larger set of their species sounds than the ones they ultimately select for their song. For example, swamp sparrows, during their learning process sing around 12 song elements, but for their final song they select 3 or 4 (Marler & Peters, 1977). In the same way, canaries (Serinus canaria) crystallize a song every year and this song remains stable for the duration of the breeding season. Every year when the song is crystallized, new
syllables are added to it and earlier syllables disappear, however year by year their syllable repertoire gets bigger (Nottebohm & Nottebohm, 1978; Nottebohm, Nottebohm, & Crane, 1986). Studies on brown-headed cowbirds (Molothrus ater) have shown that before song crystallization males overproduce song types, but then selectively retained the song types that elicit a very rapid ‘wing stroke’ display from females (West & King, 1988). So, songbirds seem to narrow their song repertoire during song development.

Both humans and songbirds seem to have an innate ability to recognize, memorize, select and produce their species’ sounds. However, experience is needed in order to activate this knowledge and shape development of their languages, and songs, respectively. Lack of experience in the few known cases where humans were raised under conditions of social deprivation had a negative impact on their language skills. A similar observation was found in birds which, in social isolation, developed an abnormal song. (Price, 1979; Brainard & Doupe, 2002; Catchpole & Slater, 2008; Doupe & Kuhl, 2008; Marler & Zeigler, 2008; Feher, 2009). Thus, regardless of an innate capacity, humans and most songbirds need to be exposed to their species-specific vocalizations during a phase early in development or a ‘sensitive period’ in order to produce effective communication signals. For instance, the human capacity to produce and detect sounds from a new language as a native speaker is significantly reduced after early adolescence (Doupe & Kuhl, 2008). In songbirds the timing for this vocal learning plasticity varied between species (Brainard & Doupe, 2002). Songbirds can be broadly divided into “open-ended learner” species, like canaries (Serinus canaria), that can incorporate new songs or elements in their adult song repertoire throughout their life, and “close-ended learner” species, like zebra finches (Taeniopygia guttata), in which the capacity to learn is restricted to a ‘sensitive period’ early in life (Brainard & Doupe, 2002; Slater, 2003; Catchpole & Slater, 2008; Amador & Margoliash, 2011).

Another similarity between speech and birdsong development is the influence of social interactions, whereby social interaction has to precede vocalizations. Children and songbird’s first vocalizations, (babbling and sub-song respectively) progressively and through imitation of the conspecific adult sounds plus the auditory feedback from their own productions, are modified over time to resemble adult vocalizations (Goldstein,
King, & West, 2003; Doupe & Kuhl, 2008). Babbling and sub-song are a fundamental step, where the young test their articulatory capacities and discover and practice the sounds of their species (Guasti, 2004; Doupe & Kuhl, 2008). This implies that hearing others, and themselves, are essential aspects in vocal learning development. Deprivation in any of these aspects leads to abnormalities in the acquisition and maintenance of speech in humans and songs in songbirds (Brainard & Doupe, 2002; Kuhl, 2003; Nottebohm, 2005; White, 2009; MacDougall-Shackleton, 2009; Bolhuis, et al., 2010).

In sum, songbirds, like humans, base much of their communication on vocal interaction. Thus, sound recognition and sound production occupy a fundamental role in their life. The importance of songs in birdsongs, their acquisition and the neural systems that support these mechanisms are the topics of the following sections.

1.2 Birdsong Function, Acquisition and Female Song Preferences.

The main functions of birdsong are mate attraction and territory defense (Catchpole & Slater, 2008). Thus, individual variation in song can have a significant influence on reproductive success and fitness through its effects on mate selection and mate competition (Gil & Gahr, 2002; Catchpole & Slater, 2008; Taffe & Theunissen, 2011). Thus, birdsong is a sexually selected trait, but unlike many other sexually selected traits such as plumage or antlers, its development depends on vocal learning. But, how do songbirds acquire their songs? The process of song learning in songbirds is generally divided in two phases: the sensory phase, where the exposure to the song of an adult conspecific ‘tutor’ takes place and an internal representation of this song (song template) is formed; and the sensorimotor phase, where the juvenile practices singing and eventually matches its vocal output to the stored auditory memory (Konishi, 1965; Brainard & Doupe, 2002; Nottebohm, 2005; Bolhuis et al. 2006).

One well-studied case is zebra finches. In this songbird species there is a clear sexual dimorphism and only the male sings. The sensory phase for males is from day 25 to 60 after hatching. The sensorimotor phase starts from around day 40 after hatching to day 90, were they produce a sub-song that then becomes a plastic song (more similar to the
tutor song) and, finally, in normal conditions, the song becomes crystallized by around day 90 and this is the song they sing for life. (Eales, 1987; Bolhuis, Zijlstra, Den Boer-Visser, & Van der Zee, 2000; Adret, 2004; Lauay, Gerlach, Adkins-Regan, & Devoogd, 2004; Catchpole & Slater, 2008).

Figure 1. Time line song acquisition in zebra finches. DAH (Days after hatching)

This song learning process led to the auditory template hypothesis, the idea that the bird’s construction of a complex sound replica is based on a set of both genetic and environmental instructions (Konishi, 1965). Evidence for this hypothesis came from studies using deafened birds or birds raised in isolation that did not have access to proper auditory stimuli and were unable to generate a normal song (Konishi, 1965; Marler 1977). However, their songs still preserved some general features of the species-typical song, suggesting some innately- encoded song features, or an inherent song template constraint that guides songbirds learning process (Searcy et al., 1985; Marler, 1997; Bolhuis et al., 2006; Feher et al., 2009, Bolhuis et al., 2010).

For example, in the case of zebra finches the sensitive period to learn and produce the tutor song is closed early in development. However, they still learn songs for recognition and discrimination later in life. Thus song recognition memories may differ from the memory of songs that contribute to the song template for vocal learning (Riebel, Terpstra, Smallegange, Terpstra, & Bolhuis, 2002).

Consequently, it had been suggested that songbirds generate two kinds of memories in order to develop a song. First, the production memory, that can be further subdivided, into a form of declarative memory associated to the sensory period of the song, where the song template guides the motor development and is characterized for being transient; and
a form of procedural memory developed during the sensory-motor phase, where the template allows the song maintenance after song crystallization. Second, the recognition memory, that seems to be present along the entire life of the birds and allows them to distinguish relevant auditory cues (Adret, 2004).

In the case of female song preferences it is not clear if they pass through an early sensitive period of memory formation analogous to that for song production, or if their memories for preferred songs is a product of song recognition ability analogous to that males have in adulthood (Lauay et al., 2004).

Female songbirds of many species use male song as a cue for mate choice, and thus females are predicted to exhibit preferences for song features that may result in higher fitness through direct or indirect benefits (Catchpole & Slater, 2008). Such features include song complexity, song performance, and the geographic dialect of the song (Nowicki and Searcy 2005). The evolutionary processes that have resulted in female song preferences have been well studied, but an important question is how such female song preferences arise through development. As in the case of male song learning, there is evidence for both innate and learned components. Studies of preferences for song dialects and familiar songs suggest that in some species adult females base their mate selection on the auditory memory of the song that was created early in life. For example, in white-crowned sparrows (Zonotrichia leucophrys) females prefer song dialects they heard early in life over other songs (Baker 1981; MacDougall-Shackleton & MacDougall-Shackleton, 2001). Similarly, female zebra finches show preferences for their foster father’ song over their genetic father’song (Clayton, 1990) and, for their father (tutor) or mate song over novel songs (Miller, 1979a,b). Female song sparrows have also been shown to prefer songs they heard early in life over songs from the genetic population (Hernandez, Phillmore, & MacDougall-Shackleton, 2008).

The examples presented above are evidence of song preferences as experience dependent behavior. Thus, early experience seems to modulate the response to a song stimuli. However, there are also indications that song preferences can be do not seem to be modified by early acoustic experience. This is the case in female canaries, (Serinus
**canaria**, raised in isolation or in aviary conditions. Both groups showed preferences for phrases with a high syllable rate and a greater bandwidth, which suggests an innate perceptual predisposition towards those song characteristics as a preference guide (Draganoiu, Nagle, & Kreutzer, 2002). A similar situation was found in house finches, *(Carpodacus mexicanus)*, hand reared and tutored either with their local song dialect, with a distant conspecific dialect, or with no exposure to any dialect. Across all groups, females preferred the local dialect independently of their early tutoring conditions (Hernandez & MacDougall-Shackleton, 2004). Hand raised female swamp sparrows, *(Melospiza georgiana)*, in spite of their tutoring conditions, show preferences for the 3-note song syntax of their population of origin (Balaban, 1988). Similar results were found in canaries (Vallet, Beme, & Kreutzer, 1998) and chafinches, *(Fringilla coelebs)* (Riebel & Slater, 1998). Thus, song preferences of females likely develop via an interaction of experience-dependent and –independent processes. Therefore, the degree of interaction between inherited predispositions and experience seems to vary among species.

So far I have been discussing song acquisition and song preferences from a behavioral perspective, but in order to produce and recognize these learned vocalizations birds require an underlying neural system that supports: i) motor commands that lead to production of complex species-specific sounds; ii) perceptual abilities that facilitate detection, identification, selection, recognition and memorization of relevant sounds; iii) mechanisms that permit them to evaluate their auditory feedback against their internal template and allow them to correct their own vocal output (Brainard & Doupe, 2002; Catchpole & Slater, 2008). A brief description of these neural systems is the focus of the following section.

### 1.3 Vocal Learning - Neural Systems of Song Production & Perception.

As noted above vocal learners require a neural system that allows them to acquire and produce their conspecific vocalizations. Comparative studies established that non-vocal learner species are able to produce specific vocalizations, “calls”, that are usually innate. Underlying these vocalizations, lower brain structures located at the brainstem and midbrain level are required. In contrast, to produce learned vocalizations forebrain
structures are also recruited in a complex circuitry (Jügens, 2002; Jarvis, 2007; Amador & Margoliash, 2011).

1.3.1 Song-control System. In the case of songbirds, this complex circuitry is called the ‘song-control system’, a network dedicated to learning and production of songs. It includes two main pathways, the posterior descending pathway or caudal motor pathway and the anterior forebrain pathway (cortical-basal ganglia loop). The caudal motor path is crucial for song production (See Figure 2). The main nucleus is HVC (used as a proper name) that projects directly to RA, robust nucleus of the arcopallium, which projects to DM, dorsomedial nucleus of the intercollicular complex, that subsequently projects to vocal and respiratory control structures at the brain stem and then to the syrinx (the sound producing organ in birds). Lesions of structures along this path lead to permanent and intense failure in song production in adult birds. HVC also projects indirectly to the RA via the anterior forebrain pathway (AFP); that is, Area X, DLM (dorsal lateral nucleus of the medial thalamus) and LMAN (lateral magnocellular nucleus of the anterior nidopallium) in a way comparable with the mammalian pathway cortex - basal ganglia – thalamus - cortex. The AFP is necessary for song learning, song modification and song control supported by auditory feedback. Hence, lesions in this path lead to interruptions of song acquisition in juveniles, but lesions have limited effects on adults after the song is acquired or crystallized (Nottebohm, 2005; Catchpole & Slater, 2008; Bolhuis & Gahr, 2006; Jarvis, 2007; MacDougall-Shackleton, 2009; Amador & Margoliash, 2011).
The “song system” (white ovals) includes: The posterior vocal pathway is composed of the ventral motor pathway which includes Uva, and Nif. The descending motor pathway (dashed lines) contains HVC, RA, DM and the nuclei involved in vocal respiratory control. The Anterior forebrain pathway or (cortical-basal ganglia loop) contains Area X; DLM, and LMAN. The Auditory nuclei (gray colored) provide inputs to the song system to HVC, Nif, and Uva. The ascending auditory pathway contains the nuclei MLd, (located in the midbrain); Ov (thalamic structure) and Field L, NCM, CMM, CLM, and CSt in the telencephalon. Diagram modified from Amador & Margoliash, (2011). See acronyms in the text or appendices Table of Abbreviations.

1.3.2 Auditory projections to the song control system. (See Figure 2) The song control system obtains auditory input through HVC, which receives projections from the thalamic nuclei Uva (nucleus uvaeformis) and from auditory forebrain structures CLM,
and possibly from the Field L directly and indirectly through NI\textsubscript{f}, nucleus interfacialis of the nidopallium, which is considered the main source of auditory input to HVC. Uva obtains information from the auditory system and PA\textsubscript{m} (nucleus paramambigualis) in the ventrolateral medulla that innervates NI\textsubscript{f} and HVC (Amador & Margoliash, 2011).

1.3.3 Auditory System. The general organization of the auditory pathway in songbirds follows the same pattern of the mammalian auditory system, being an ascending pathway. (See Figures 2 and 3) Auditory information goes from the cochlea to the auditory branch of the XVIII cranial nerve, and then ascends to the brain through the midbrain nuclei, ML\textsubscript{d} (mesencephalicus lateralis pars dorsalis), homologous to the inferior colliculus in mammals, then to the auditory thalamic structure O\textsubscript{v} (nucleus ovoidalis), homologous to the medial geniculate nucleus in mammals. This thalamic structure projects to a thalamo-recipient zone in the telencephalon called Field L\textsubscript{2}, which is a dense granular cell layer (Layer IV primary auditory cortex in mammals) that contains reciprocal projections to Fields L\textsubscript{1} and L\textsubscript{3}. The Field L, as a totality, sends an intricate set of projections to NCM (caudomedial nidopallium) CMM (caudomedial mesopallium), CL\textsubscript{M} (caudolateral mesopallium), and CSt (caudal striatum). CL\textsubscript{M} shares reciprocal connections with the different components of Field L. Field L\textsubscript{3} sends projections to NCM. NCM and CL\textsubscript{M} project reciprocally to CMM. NCM, CMM and CL\textsubscript{M} are considered secondary auditory structures, because of their suggested role in perceptual processing and discrimination of complex auditory stimuli, such as vocal communication signals, and associative learning that contain auditory cues (Mello & Pinaud, 2006; Catchpole & Slater, 2008; Amador & Margoliash, 2011).
Electrophysiological studies suggest that the auditory pathway processes sensory information, including conspecific vocalization in a hierarchical level of specialization, such that the information rises from the peripheral areas to the higher-level of processing at the forebrain (Amin, Grace, & Theunissen, 2004). At the forebrain level field L is considered the first specification level, being able to identify between heterospecific songs and synthetic songs over conspecific songs. The following level involves CM and CNM. CM exhibits greater selectivity for vocalizations compared to synthetic sounds (Adret, 2004; Amador & Margoliash, 2011; Taffeta & Theunissen, 2011). Its role has also been associated with processing behaviorally relevant sound. Indeed, lesions in CM of female zebra finches leads to failure in the normal responses to conspecific and heterospecific song (MacDougall-Shackleton, Hulse, & Ball, 1998). Neurons from CM in
European starlings (using electrophysiological recordings) show that their selectivity properties are largely dependent on the bird’s experience and perceptual learning can modify their selectivity (Gentner & Margoliash, 2003). NCM in zebra finches also shows a hierarchical organization, being more responsive to conspecific song, than to heterospecific or non-auditory stimuli and little or no response to the presentation of white noise or tone stimuli (Mello, Vicario, & Clayton, 1992).

The variability in immediate-early gene (IEG) in brain regions outside of the song system (such as NCM and CMM) in songbirds (canaries and zebra finches) discovered by Mello et al. (1992), has directed the interest of researchers toward understanding the possible functional associations that can be involved in song production, learning and perception. Since then, the measurement of IEG has become the standard technique to assess neural depolarization during exposure to a stimulus in the avian brain (MacDougall-Shackleton, 2011). Therefore, neural activation, as assessed by IEGs, in NCM and CMM is the focus of my research. The following section provides a brief review of IEGs, specifically ZENK, as well as the possible functionality of NCM and CMM associated with the levels of IEG expression.

1.4 IEGs - Definition & Evidence for the Functionality of Auditory Forebrain Regions.

IEGs (or primary response genes) are a class of genes, that are rapidly and transiently expressed in response to a variety of cellular stimuli, ranging from external stimulation that leads to neuronal depolarization to chemical stimulation (Mello, 2002; Pinaud, 2005; Terleph & Tremere, 2006). In avian research, one of the most commonly used IEGs is the nerve growth factor induced gene-A (NGFI-A, also known as, zif-268, egr-1, ngf-Ia and krox-24, zenk), (Here after ZENK). ZENK encodes a transcription factor that is found in the promoter of different genes expressed within the nervous system, and it seems to have an important role in the control of neural plasticity mechanisms (Pinaud, 2005). ZENK seems to be part of an early molecular regulatory cascade of events, generated by extracellular stimulation. This cascade involves continued depolarization, and it is mainly coordinated by intracellular calcium (Ca^{2+}) influx, as a result of N-methyl-D-aspartate (NMDA-type) glutamatergic receptor activation. This NMDA activation leads to the
opening of voltage sensitive \( \text{Ca}^{2+} \) channels. The consequent influx of \( \text{Ca}^{2+} \) regulates an intracellular flow of biochemical events that culminate in the induction of IEG, in this case, ZENK expression (Ribeiro & Mello, 2002; Pinaud, 2005; Terleph & Tremere, 2006). The identification of the products that result from the ZENK expression (mRNA or ZENK protein) allows the detection of the cells that were depolarized during the exposition to the external stimuli (Pinaud, 2005).

Even though the exact relationship between neuronal activation and ZENK expression has not been completely understood, the analysis of induced expression of IEGs has had an important role in the identification and study of brain regions activated by specific sensory stimuli or behavioral conditions. In fact, lack of sensory stimulation leads to low or moderate basal ZENK levels, and particular kinds of stimulation generate high levels of ZENK activation. Additionally, its expression seems to indicate the places where the experience-dependent changes take place (Mello & Pinaud, 2006; Terleph & Tremere, 2006). Consequently, ZENK has become a brain-mapping tool for detection of event-triggered or behaviorally-triggered regional brain activation (Mello, 2002). Indeed, many of the forebrain areas that process auditory stimulation were initially identified by ZENK expression (Maney, MacDougall-Shackleton, MacDougall-Shackleton, Ball, & Hahn, 2003). A pioneering study in the use of ZENK expression in songbird’s auditory forebrain areas exposed male canaries and male zebra finches to conspecific songs, heterospecific songs, tone burst, or silence. After the stimuli presentation, a significantly higher expression of ZENK in NCM and CMM in birds exposed to the playback of conspecific song as opposed to the other stimuli was found. It was concluded that meaningful natural stimuli may quickly induce ZENK to higher levels (Mello et al., 1992). Additionally, this elevated neuronal activation in NCM and CMM, following the presentation of novel conspecific song, was not found in song control system nuclei (such as, HVC, Area X, LMAN or RA) of songbirds that are non-singing. However, singing birds that do not hear songs show high ZENK expression in HVC, but not in NCM or CMM. Differentiation in ZENK expression suggests that there is a functional dissociation between forebrain regions that are activated when the bird hears song, and when it is singing itself (Jarvis & Nottebohm, 1997).
As discussed previously, NCM and CMM are considered to play an important role in perception and processing of complex auditory stimuli. Additionally, the variability in ZENK expression in these areas in response to different stimuli has motivated the study of using this technique in different species. Recent evidence seems to support a relation between ZENK expression in NCM and CMM with song preferences. Thus, females appear to respond selectively to songs that are contextually relevant (Maney et al., 2003). NCM ZENK expression in female budgerigars, \textit{(Melopsittacus undulates}, a non-songbird), exposed to either standard male song, complex song, or simple song show higher levels of activation associated to complex song (behaviorally preferred) (Eda-Fujiwara, Satoh, Bolhuis, & Kimura, 2003).

Female white-crowned sparrows were exposed to conspecific male song of either local (preferred) or foreign dialect. ZENK response in CMM and NCMD was positively correlated with the local dialect and the number of sexual displays performed in response to song (Maney et al., 2003). Similarly, female European starlings were exposed to conspecific short or long songs (preferred). ZENK expression in NCM was higher in response to long songs. However, this differentiation was not found in CMM where ZENK expression was consistently high across subjects (Gentner, Hulse, Duffy, & Ball, 2001).

Therefore, in some species of songbirds, female preferences vary depending on the nature of the song stimuli (such as, length, complexity, familiarity, etc. discussed in 1.2), and this variation seems to be reflected by the levels of ZENK expression in CMM and NCM. Thus, NCM and CMM activation appears to respond to the biological salience of the song stimuli (MacDougall-Shackleton, 2011). Nevertheless, explanations that account for this apparent association between ZENK variability and the nature of the song stimuli are not entirely understood. Evidence presented by different research leads to a different interpretation of variability in ZENK expression. One interpretation of variation in ZENK response to song is that it results from the relationship of the heard stimulus and the \textit{memory of the tutor song}. The rationale that supports this perspective came from different observations. For example, IEG expression
in NCM in male zebra finches appears to be correlated positively with the accuracy or strength of the song copy made from the tutor song (measured as the number of elements copied). Additionally, CMM seem to be implicated in encoding song characteristics such as the length of the song (Bolhuis, Hetebrij, Den Boer-Visser, De Groot, & Zijlstra, 2001). In another study female zebra finches reared with their father showed significant preference for their father’s song. This preference was related to high levels of ZENK activation in CMM (Terpstra, Bolhuis, Riebel, Van der Burg, & Den Boer-Visser, 2006).

Juvenile zebra finches show an increasing level of ZENK expression in NCM during the sensorimotor learning phase. So, ZENK expression was higher during the later phase in response to the tutor song compared to the earlier phase. (Gobes, Zandbergen, & Bolhuis, 2010). Additionally, a significant positive correlation was found between ZENK activation in NCM in response to the tutor song as opposed to the bird’s own song or a novel song (Terpstra, Bolhuis, & den Boer-Visser, 2004). Based on this evidence, it is has been proposed that NCM is, or makes part of, the neural substrate for the representation of the tutor song (Bolhuis & Gahr, 2006).

A second interpretation of variation in ZENK responses to song proposes that ZENK activation in NCM and CMM reflect the biological salience of the song, and result from increased perceptual processing through some kind of attentional mechanisms (MacDougall-Shackleton, 2001). For instance, ZENK expression in forebrain auditory areas decreases after repeated exposition to the same song via habituation. However, the ZENK activation levels increase following the presentation of a novel song (Mello, Nottebohm, & Clayton, 1995). In a series of experiments, male zebra finches were exposed to repetitions of a song until reaching habituation. Later, the same song was presented again but in one of four different contexts (speaker from a different side, reduced sound pressure level, paired with illumination, and paired with colored illumination). This second presentation generated increased levels of ZENK activation in NCM and CMM (Kruse, Stripling, & Clayton, 2004). Similarly song sparrows exposed to novel songs show higher levels of ZENK activation in NCM in contrast to those that were exposed to the same song (McKenzie, Hernandez, & MacDougall-Shackleton, 2006). In these studies, then, higher ZENK responses were associated with increased attention or salience, rather than a similarity to the bird’s auditory memory.
In summary, two hypotheses have been proposed to explain the correlation between variation in song playback and variation in the levels of ZENK activation in NCM and CMM. 1. ZENK activation in NCM and CMM reflects the access to a representation of tutor song memory. Therefore NCM and CMM are the neural substrate for the memory of the tutor song acquired during the sensory phase. 2. ZENK activation in NCM and CMM reveals attentional mechanisms. Of course, these two hypotheses are not entirely mutually exclusive, as songs that are similar to a stored song memory may capture more attention or have greater perceptual processing. However, the second hypothesis posits that these attentional mechanisms can operate regardless of the tutor song memory.

The aim of my thesis is to clarify the nature of ZENK responses and to test the two hypotheses above. I propose that if there is a representation of a bird’s tutor song (template) encoded in the NCM or CMM, I will observe more ZENK activation in the experimental groups in response to playback of their tutor song compared to other stimuli. However, if the ZENK response in the NCM and CMM reflects attentional mechanisms, I will obtain more ZENK activation across the groups in response to the playback of their conspecific song, compared to other stimuli. I use a novel approach of tutoring female zebra finches with song from isolate-reared males and with another species. The rationale for this approach is that wild type zebra finch song may capture more attention of females, even if they were tutored with another species, or an isolate-male’s songs. Thus, if ZENK activation reflects song memories I should observe higher response to tutor song, and if ZENK activation reflects attention I may observe higher response to wild type song regardless of tutoring experience.
Chapter 2

2 Materials and Methods

I examined Zenk immunoreactivity (ZENK-ir) in three auditory forebrain areas of female zebra finches (*Taeniopygia guttata*) raised in different tutoring conditions. The ZENK induction was measured following the presentation of songs that did, or did not, match the birds’ prior experience (See below).

2.1 Animal procedures

Zebra finches from the aviary colony maintained at the Advanced Facility for Avian Research (AFAR) at the University of Western Ontario, London, Ontario, Canada were used as parents for the experimental subjects.

For breeding purposes a female and a male were paired in individual breeding cages. Two plastic containers were provided as nest cups. One of them contained wood bedding and the other one contained hay. The birds selected one of them to build their nests. Birds had access to multi-vitamin seeds, grit and cuttlefish bone and water *ad libitum*. One spoon of egg-food mix (blended hard-boiled egg and bread) was provided daily during the breeding process, when the chicks hatched the amount of egg food mix was increased to one tablespoon per chick. This mix was provided until the offspring were moved to the experimental groups. The light cycle was 14 h light: 10 h dark during the experiment. Rooms were maintained at 24° C.

From the breeding pairs 58 female zebra finch offspring were obtained, they were raised by both parents until day 10 post-hatch of the first chick in the clutch. At day 10 the father was removed from the cage to avoid the chicks hearing their father’s song. The mothers and the chicks were moved to a room with other females (who do not sing) and their chicks. Zebra finches typically do not learn songs heard previous to day 25 after hatching, and they require at least 10 days of interaction with the father to make an accurate copy of the song (Roper & Zann, 2006). Additionally parental recognition seems to be complete by day 16 after hatching (Lassek & Bischof, 1985).
To determine the sex of the birds, small blood samples were collected and DNA extracted. The sex was then determined through PCR amplification of genes located on the sex chromosomes (Griffiths, Double, Orr, & Dawson, 1998).

The mother raised the chicks until the median age of the clutch was 35 days after hatch, when offspring could feed independently (Clayton, 1987). At that day, birds were randomly assigned to one of the experimental groups (See Figure 4).

**Figure 4. Time line birds manipulation.**

2.2 Experimental groups – Description

Female zebra finches were randomly assigned to one of three tutoring condition groups. Hence, each group was exposed to different early auditory experiences from day 35 after hatching until maturation around day 90.

2.2.1 Group 1. Wild Type Conspecific tutored. This group was composed of 19 zebra finch females that were tutored by one of seven wild-type conspecifics, which means that they were exposed to zebra finch song.

Zebra finch song is highly stereotyped, around 3-10 seconds in duration, and contains a sequence of one repeated motif or phrase (Figure 5). It has different types of syllables that are mostly composed of harmonically related tones in a range of 0.3-4.2 kHz. These syllables normally appear in a fixed number and sequential order (Watanabe & Sakaguchi, 2010)
Figure 5. Spectrograms of two wild-type zebra finches songs.

2.2.2. Group 2. Isolate conspecific tutored. This group was composed of 20 female zebra finches that were tutored by one of seven male zebra finches that had an isolate song. Throughout their song-learning period (day 10-90) these males were kept in social and acoustic isolation, as a result they developed an ‘isolate’, ‘autogenous’ or ‘untutored’ song (Williams, Kilander, & Sotanski, 1993). Hereafter I refer to these songs as isolate song.

Isolate songs (Figure 6) are simpler and more uniform in structure than wild-type song, composed of fewer notes per syllable and per song. The syllables tend to be higher in frequency and longer in duration than those of wild-type song. This song is also characterized as being less rhythmic, scratchier, and more monotonic compared with wild-type zebra finch songs (Price, 1979; Searcy et al., 1985; Feher, 2009). In spite of this, zebra finch isolate songs have some characteristics of normal wild-type zebra finch songs. They are initiated by a string of introductory notes that include four to twelve stereotyped syllables, many of which can have normal harmonic structure (Price, 1979).
Figure 6. Spectrograms of two Isolate conspecific zebra finch songs

To obtain the isolate songs, seven isolated tutors were breed from the wild type zebra finches. The breeding conditions were the same used to obtain the females for this experiment, however, when the young males reach independency around day 35 after hatching, they were individually housed in a soundproof isolation chamber (wide 91cm X deep 71cm X height 172cm) until day 140 after hatching. Song development in zebra finches is completed by 4 months after hatching (Price, 1979).

It has been suggested that isolate song is created from the limited auditory input the isolate bird experienced, primarily its own vocal output (Williams et al., 1993). The isolate bird then rejects or includes sounds in its song depending on how appropriate they match in the ‘innate template’ for its species’ song. In this song development process there is not any effort to imitate a model, because there is not an external model to be acquired (Williams et al., 1993).
2.2.3 **Group 3. Wild Type Heterospecific tutored.** This group was composed of 19 female zebra finches that were tutored by one of five heterospecific songbirds, specifically Bengalese finches (*Lonchura striata*). This means that females from this group were exposed to a wild type heterospecific song.

Bengalese finches were selected because there is evidence that zebra finches tutored by Bengalese finches are able to make an accurate copy of the Bengalese finch song, even though it is not their species song (Clayton, 1988). Additionally, zebra finches and Bengalese finches are closely related (Family Estrildidae), and share neural structures dedicated to acquisition and production of song (Zeng, Székely, Zhang, Lu, Liu, & Zuo, 2007; Watanabe & Sakaguchi, 2010). Bengalese finch syllable syntax and syllable types rarely occur in normal wild-type zebra finch songs (Funabiki & Konishi, 2003).

Similar to zebra finches every Bengalese finch male has an individual song. This song has a duration around 10 – 20 seconds, and is composed of one repeated motif (Figure 7). Every motif consist of several syllables that are largely composed of harmonically related tones from a range between 0.2 – 5.0 kHz. Bengalese finch song tends to have a stereotyped sequential order. In spite of this, the number of syllables can vary within a motif (Clayton, 1987; Watanabe et al., 2010). Even though both zebra finches and Bengalese finches sing a stereotyped song Bengalese finch song appears to be less stereotyped in syllable organization and tends to be more complex than the zebra finch song (Watanabe et al., 2010). Syntactically, Bengalese finch songs are characterized by syllable repetition (such as aaabbccccc), which differs with the zebra finch song that maintains a fixed sequence of syllable (such as abc) (Funabiki et al., 2003).
Figure 7. Spectrograms of fragments of two wild type Bengalese finch songs

The five wild type Bengalese finch tutors for this experiment were purchased from a breeder and had been reared with Bengalese finch parents.

All experimental groups were composed of subgroups of 1 tutor male and 2 or 3 young females housed in the same cage. Birds from the same group had visual and auditory access to other cages from the same group, but they didn’t have any opportunity to interact with tutors or tutees from other cages. The distance between the cages was at least 50 cm. Therefore, it is unlikely that the proximity to other cages interfered in the song tutor selection because the lack of free social interaction reduces the suitability of the tutor (Eales, 1986). Moreover, male zebra finches copy the song from tutors that they had social interaction with and that behave more aggressively towards them, rather than birds in adjacent cages (Clayton, 1988). During the experiment there was no visual or auditory contact between groups. Every group was housed in different rooms. Every
subgroup remained together until females reach maturity, around day 90.

2.3 Playback Procedure

When females reached maturity (after day 90 after hatching), they were randomly assigned to one of five subgroups depending on the song playback to which they were exposed: Tutor song (which is differentiated as the song produced by the rearing male), wild-type conspecific song, wild-type heterospecific song, isolated conspecific song, or white noise.

![Figure 8. Time line Playback Procedure](image)

### Table 1. Number of Birds per Group and Playbacks distribution.

<table>
<thead>
<tr>
<th>PLAYBACKS</th>
<th>Wild Type Conspecific Tutored</th>
<th>Isolate Conspecific Tutored</th>
<th>Wild Type Heterospecific Tutored</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>Heterospecific</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Song</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>Isolate</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>Conspecific</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Song</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>Tutor</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>Song (Non-tutor)</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>White Noise</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>20</td>
<td>19</td>
<td>58</td>
</tr>
</tbody>
</table>

All the songs used as stimuli were obtained from the different tutors (Isolated conspecific, wild-type conspecific, wild-type heterospecific Bengalese finch, and tutor song) described above. To record the stimuli, each bird was isolated in a soundproof isolation chamber for around 20 h; following this isolation period a conspecific female in a different cage was introduced in the soundproof isolation chamber to stimulate the male to sing. Therefore, all songs used were female-direct songs. Songs were recorded for 10
minutes using an omni-directional microphone (Sennheiser ME62/K6P) and a digital audio recorder (MARANTZ PMD671) with a sampling rate of 44.1 kHz and 16-bit resolution. Birds were recorded twice with an interval of at least 7 days and all the birds were older than 4 months, so their song was already crystallized. Zebra finches and Bengalese finches have one song that they repeat during their lifetime. Using sound analysis software Raven pro (Cornell Lab of Ornithology) the recordings of each bird were examined and one of the directed songs was selected for use as a stimulus. This song was repeated in 30-second intervals, having one-second interval between song repetitions, followed by 30 seconds of silence. Therefore, each minute of playback corresponds to 30 seconds of exposure to the song playback and 30 seconds of silence. Depending on the song duration, the song was repeated two, three or four times until reaching the 30 seconds of song playback. This process was repeated to produce 19 playback song stimuli (7 wild type zebra finch, 7 Isolate zebra finch and 5 Bengalese finch).

For the white noise playback, a white noise mp3 file from an Internet website (http://whitenoisemp3s.com) was downloaded and played in the same isolation chamber. The white noise playback was then recorded with the same equipment as above. The stimulus was then treated as the other playbacks. White noise segments were 30 seconds in duration and arranged in two repetitions of 15 seconds, having one-second interval between white noise repetitions, followed by 30 seconds of silence.

Prior to the playback exposure each female bird (see section 2.2 above) was moved into a 36.5 cm X 24 cm X 30 cm cage that contained one seed cup, one water bottle, one grit cup, a cuttlebone and two perches (food and water were provided ad libidum) inside a sound proof isolation chamber. Each isolation chamber was equipped with 2 playback speakers (KOSS HDM/111 BK) used to broadcast the stimuli. Speakers were placed in front and at the side of the cage. The volume of the speakers was adjusted to produce an average sound intensity of 75 dB SPL at the position of the cage.

Birds were kept in complete isolation for approximately 24 hours. 15 minutes before the stimuli was played the lights inside the soundproof isolation chambers were turned off to
avoid as much as possible any movement or vocalization that can cause IEG expression and can confound the experiment. The randomly assigned song playback was repeated for 30 minutes. One hour after the stimulus was played, while still in the dark, birds were given an overdose of isoflurane anesthetic, decapitated and the brains were rapidly dissected from the skulls.

All the experimental procedures, care, and housing conditions were conducted according to and with the approval of the University of Western Ontario’s animal use regulations.

2.4 Tissue preparation and Immunocytochemistry

Once brains were collected they were immediately immersed in 4% paraformaldehyde for at least 4 days to fix them. Fixed brains were immersed in 30% sucrose for 48 h at 4°C to cryoprotect them, then the brains were frozen rapidly in powdered dry ice before storage at -80°C until processing.

Brains were sliced in 40-µm thickness in parasagittal sections starting from the midline on a cryostat. Every second section was collected into 0.1 M phosphate buffered saline (PBS). Sections were immunolabeled to localize ZENK protein (egr-1) as follows: free floating sections were washed twice in 0.1 M PBS, incubated for 15 minutes in 0.5% H2O2 at room temperature, then washed three times in 0.1 M PBS. Then sections were blocked using 10% normal goat serum (Vector labs, catalog # S-1000) in 0.3% Triton-X 100 (PBST) for 1 h incubation at room temperature. The normal goat serum was then removed and the sections were incubated for approximately 20 h at 4°C in the primary antibody, a polyclonal antibody reared in rabbit (Egr-1, Santa Cruz Biotechnology, catalog # Sc-189) at a concentration of 1:2000 in 0.3% PBST. Sections were then washed three times in 0.1% PBST and incubated for 1 h at room temperature in the secondary antibody, biotinylated goat anti-rabbit, (IgG, Vector labs, catalog # BA-1000) 1:250 diluted in 0.3% PBST. After that, sections were washed in 0.1% PBST three times and incubated in avidin-biotin horseradish peroxidase complex (ABC Vectastain Elite kit, Vector labs, catalog # PK-6100) at room temperature for 1 h. Then sections were washed in 0.1% PBST and visualized using 3’,3-diaminobenzidine tetrahydrochloride chromagen (Sigma Fast DAB) and washed three times in PBS. Brains were processed in groups of 3
to 4 and each immunohistochemistry run had brains from the different treatment groups. Finally sections were mounted on gelatin-coated microscope slides, dehydrated in ethanol and cleared in solvent (Harleco Neo-Clear, EMD Chemicals) and protected with coverslips affixed with Permount (Fisher Scientific).

2.5 ZENK quantification

The level of Zenk immunoreactivity (ZENK-ir) was quantified in three forebrain auditory areas within the telencephalon: the dorsal caudal medial nidopallium (NCMD), the ventral caudal medial nidopallium (NCMV) and the caudal medial mesopallium (CMM). Dorsal, ventral and caudal areas of NCM boundaries were defined taking the lateral ventricle as a reference point (Figure 9). NCM rostral border area was defined using Field L that was visible as an area without immunoreactivity. CMM was delineated by the most caudal area bounded by the lateral ventricle and the caudo-ventral border of the mesopallial lamina. Six sections of one hemisphere of each zebra finch were measured. Quantification started with the first section, moving medial to lateral in which NCM was attached to the rest of the brain. Therefore, six photomicrographs per area, per bird were taken. For NCMD the photomicrographs were taken from the most dorso-caudal part of NCM. NCMV photomicrographs were obtained from the center of the ventro-rostral area. CMM photomicrographs were acquired from the most caudal part of the structure. In all three forebrain auditory regions we capture images from the areas with the highest density of immuno-positive ZENK cells within the area. This sampling procedure replicates that used in numerous previous studies (Gentner et al., 2000; Hernandez & MacDougall-Shackleton, 2004; Avey, Phillmore, & MacDougall-Shackleton, 2005; Schmidt, McCallum, MacDougall-Shackleton, & MacDougall-Shackleton, 2013).
Figure 9. (a) Sagittal section of the female zebra finch brain showing the auditory forebrain regions where Zenk-ir was quantified. (b) The approximate areas sampled within each region CMM = caudomedial mesopallium, NCMD = dorsal caudomedial mesopallium, and NCMV = ventral caudomedial mesopallium and Field L2 that exemplified an area where immunoreactivity is absent. Rostral is to the right, and dorsal is to the top. The three boxes below are miniature versions of the pictures taken when the region shows high, low or none activation.

Images of each area (0.515 X 0.386 mm) were captured using a Leica Digital CCD
camera mounted on a Leica DM5000B light microscope through a X20 objective lens (Leica Microsystems, Richmond Hill, ON, Canada). We used Leica Application Suite to compile each picture as a z-stack from a series of images taken at a regular interval (0.63 mm) throughout the focal depth of the section using a Leica 420D camera. Compiling these photomicrographs created an image in which all cells were in focus. (Hall & MacDougall-Shackleton, 2012). The observer was blind to the group treatment and to the playback broadcasted to the bird.

For each image, we used ImageJ64 (NIH) software to count the number of ZENK-ir cells in the whole image. First we converted the images to 8-bit gray scale, after that the number of particles with an optical density above a threshold value were counted using the threshold tool. This threshold was set manually in every image due to the variability in the background staining, in a way that the group of pixels emphasized by the software were equivalent with what a blind observer considered labeled nuclei, To set exclusion limits for cell size (2.0 – 56 µm²) we randomly selected 6 birds and from the 18 photomicrographs per bird (6 x each area) and choose a subset of 20 cells. So from the cells subset (360 measurements per bird, 2,160 measurements in total) we determined the minimum and maximum sizes of the cells and established a minimum and maximum. Exclusion limits for sphericity were set at 0.45. The observer was blind to the bird’s tutoring condition and to the song playback broadcasted to the subject.

2.6 Statistical analysis.

Statistical analyses (GLM) were performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). I conducted a 3 (brain auditory region) x 3 (tutoring) x 5 (playback) design. The purpose of this experiment was to evaluate the number of ZENK-ir cells in the different auditory brain areas (CMM, NCMD, NCMV) with respect to the tutoring conditions and late responses to song stimuli. The tutoring conditions represented early experiences and there were 3 between-subject tutoring conditions: wild-type conspecific tutored, isolate conspecific tutored and wild-type heterospecific tutored. There were 5 between-subject conditions of song playback stimuli: wild type conspecific song, isolate conspecific song, wild type heterospecific song, tutor song, and white noise. Number of cells activated by the ZENK-ir in three different auditory areas (CMM, NCMD, NCMV) represented the
within-subjects factor of brain auditory region.

Given that there can be interactions between lateralization and brain regions, I also investigated whether there was an effect of lateralization on the number of ZENK-ir cells in the different auditory brain areas (CMM, NCMD, NCMV) by using a 2 (hemisphere) x 3 (brain auditory region) mixed factorial ANOVA (Moorman et al., 2012). Once we established that lateralization had a significant effect, the data were analyzed using mixed factorial ANOVA with Greenhouse Geisser correction for violations of the sphericity assumption. Holm-Bonferroni corrections were used for all pair wise t-test comparisons.
Chapter 3

3 Results.

First, I will present the effects of lateralization, followed by the main effects and interactions of tutoring and playback conditions on ZENK ir cells.

There was no interaction between brain region and hemisphere on the number of ZENK-ir cells, $F(1.72, 96.34) = 0.29$, ns, and hemisphere had no effect, $F(1, 56) = 0.00$, ns. Consequently, hemisphere was omitted as a factor for subsequent analyses.

Although the main effect of tutoring was not significant, $F(2, 43) = 0.34$, $p > 0.70$, nor was the effect of playback, $F(4, 43) = 2.06$, $p > 0.10$, there was a significant main effect of brain region on ZENK-ir, $F(1.77, 75.99) = 68.06$, $p < 0.001$, $\eta^2_p = 0.61$. Using repeated measures $t$-tests, the ZENK-ir cell count in CMM was the highest of the three brain regions ($M = 317.32$, $SD = 160.32$; see Figure 10) as it was significantly greater than that in NCMD ($M = 238.70$, $SD = 136.44$; see Figure 10), $t(57) = 7.65$, $p < 0.001$, and NCMV ($M = 182.88$, $SD = 108.57$; see Figure 6), $t(57) = 9.72$, $p < 0.001$. The cell count in NCMD was significantly greater than that in NCMV, $t(57) = 5.24$, $p < 0.001$.

![Figure 10. Number of ZENK-ir cell count on the different auditory brain regions. Error bars are ± 1 SE](image-url)
There was also a significant interaction between brain region and playback, $F(7.07, 75.99) = 2.39, p < 0.05, \eta^2_p = 0.18$ indicating that the variation in Zenk response across playback stimuli varied across brain regions. One-way ANOVAs revealed that there was a marginally significant effect of playback on the number of ZENK-ir cells in CMM, $F(4, 53) = 2.49, p < 0.06, \eta^2_p = 0.16$, (see Figure 11) and NCMD, $F(4, 53) = 2.38, p < 0.07, \eta^2_p = 0.15$ (see Figure 12); however, there was no effect playback on NCMV, $F(4, 53) = 0.68, ns$ (see Figure 13).

![Figure 11. Number of ZENK-ir cell count on CMM in response to playback. Error bars are ± 1 SE](image)
Figure 12. Number of ZENK-ir cell count on NCMD in response to playback. Error bars are ± 1 SE

Figure 13. Number of ZENK-ir cell count on NCMV in response to playback. Error bars are ± 1 SE
Contrary to predictions, there was no interaction between brain region, tutoring, and playback on ZENK-ir cell count, $F(14.14, 75.99) = 0.89$, $ns$. In addition, neither the interaction between brain region and tutoring, $F(3.53, 75.99) = 0.26$, $ns$, nor the interaction between tutoring and playback were significant, $F(8, 43) = 0.81$, $ns$.

It is possible that the absence of significant differences in ZENK reactivity in response to the different song playbacks and rearing conditions was a result of lack of statistical power. However, my experiment had similar sample sizes to previous research that did detect significant differences (e.g., Mello et al., 1992; Hernandez et al., 2004). Sensitivity power analysis using G power analysis (Faul, Erdfelder, Lang, & Buchner, 2007) showed that in this study I could detect an effect size $f = 0.351$, that is considered a medium to large effect (Cohen, 1969). Thus, the null results observed indicate either no difference or only a small difference between groups.
Chapter 4

4 Discussion

In this experiment, female zebra finches showed different levels of neuronal activation across three auditory forebrain regions CMM, NCMD, and NCMV. This variation seems to depend on the auditory stimuli the birds were exposed to, where CMM and NCMD show a trend of ZENK activation that was not found in NCMV. Interestingly, there was not a significant main effect between the playback songs and the ZENK expression patterns, which indicates that the different playbacks equally stimulated CMM and NCMD in these females. Therefore, it appears that as long as birds are exposed to complex sounds they will have enhanced levels of ZENK expression in their auditory areas. Furthermore, this variation does not seem to be associated with early tutoring conditions. Consequently, the female zebra finches of this experiment show different neuronal activation in CMM and NCMD in response to complex sounds, and this activation appears not to be affected by early acoustic exposure.

This finding is in accordance with results from Hernandez & MacDougall-Shackleton, (2004) where female house finches did not show differential patterns of Zenk expression in auditory forebrain regions to songs heard early in life over novel songs. The ZENK activation trend did not relate with early experiences. Consequently, the ZENK responses to the stimuli heard late in life seem to have little influence from early auditory experiences.

However, Hernandez & MacDougall-Shackleton, (2004) did not find a positive correlation between the levels of neuronal activation and the results from the behavioral preference task (see 1.2) that have been found in studies with other species of birds, (budgerigars, Eda-Fujiwara et al., 2003; white-crowned sparrows, Maney et al., 2003; European starlings, Gentner et al., 2001 see 1.4). Nevertheless, as Hernandez & MacDougall-Shackleton, (2004) pointed out, the ZENK activation levels could be reflecting the behavioral salience of the song rather than the auditory memory of the song, even if they do not correlate with the behavioral preference. This proposition could be supported by the production memories (or an analog for females) and recognition
memory suggested by Adret (2004 See section 1.2), and the degree of interaction between early experiences, inherited predispositions shaping the song preferences (Nagle & Kreutzer, 1997; Draganoiu et al., 2002). Further research is needed to elucidate this correlation between ZENK activation and experience – dependent and independent song preferences.

The proposal that ZENK responses to song stimuli are little influenced by early auditory experiences seems to be supported by my findings. Thus, even though I did not find an interaction between early experiences and ZENK expression, I did find an interaction between ZENK expression in the different auditory brain regions, (specifically, CMM and NCMD) and the playback that they were exposed to late in life. Therefore, it seems that independent of the auditory experiences, zebra finch females show different levels of neuronal activation in CMM and NCMD in response to different auditory stimuli presented in adulthood. Thus, the activation in the three areas depends on the playback stimulus heard. This effect is seen in CMM, followed by NCMD, but does not seem to be true for NCMV. Zenk induction appears higher for all of the song playbacks compared to white noise in CMM and NCMD. Similar results were found by Ribeiro et al., (1998) whose work with canaries showed that ‘natural’ sounds produced clusters of ZENK activation, but that the farther the stimuli went away from ‘natural’ sounds, the broader the activation in the auditory regions. As the stimuli became more synthetic, the activation, over a wider spectrum, was higher. Whereas ‘natural’ sounds produced activation in a specific area and of a specific type, synthetic sounds did not show as much discrimination (Ribeiro, Cecchi, Magnusco, & Mello, 1998).

The results did not show significant differences in activation between the different playbacks as in Mello et al., 1992). This means that the females in this experiment did not respond significantly more to either conspecific song, isolate song, or heterospecific song. This can possibly be explained by the fact the four playback song stimuli (Wild type conspecific song, isolate conspecific song, wild type heterospecific song and tutor song) that the females were exposed to share many acoustic features. All of them start with introductory notes, their syllables are in a similar frequency range, and they are mainly composed of harmonic tones (Price, 1979; Clayton, 1987; Watanabe et al., 2010
See section 2.2). Bengalese finch and zebra finch song are different particularly at the syntactic level, Bengalese finch songs are characterized by syllable repetition (such as aaabbbccc), which differs with the zebra finch song that maintains a fixed sequence of syllable (such as abc). Also Bengalese finch songs are longer in duration (Funabiki et al., 2003 See section 2.2). It thus appears that the Zenk responses in auditory regions was similar due to similar acoustic features, despite differences in song length and syntactic structure.

It was recently proposed that ZENK expression in NCM and CMM appears to correlate with song preferences across various species (Maney, et al., 2003; Terpstra et al., 2008, MacDougall-Shackleton, 2009; Eda-Fijuwara et al., 2003; Gentner et al., 2001). This preference could be associated with the possibility that female songbirds form auditory memories early in life, based on the tutor song, and there memories are neurally represented in NCM and CMM (Bolhuis, Zijlstra, Den Boer-Visser, & Van der Zee, 2000; Bolhuis & Gahr, 2006. See section 1.4). This hypothesis, however, is not supported by my results. Overall, there was not a significant interaction between tutoring conditions, playback, and number of ZENK-ir cells expressed in CMM, NCMD, and NCMV. Contrary to Maney, et al., 2003; Terpstra et al., 2008, etc., my findings suggest that these females early tutoring experiences did not seem to influence the adult responses to the different auditory stimuli in the auditory forebrain areas. However, I did not have a parallel behavioral study that can corroborate the absence of correlation between ZENK expression in auditory brain areas and behavioral preferences.

Some alternative hypotheses that can explain the fact that my experiment did not support the neural representation of the tutor song are as follows: First, there is the possibility that the song is not represented in those areas. Therefore, as was observed, we should not expect to have significantly different activation in the auditory brain areas in response to the tutor song compared with other stimuli. (See figure 7). Second, it is possible that the activation is high only when female zebra finches are raised by their wild type conspecifics and, consequently, their tutor is a wild type zebra finch. However, that does not seem to be the case. Although the sample size is small, there appears to be no
difference in Zenk response to tutor song across the three rearing groups (See figure 14). That is, Zenk response to tutor song was not higher in wild-type reared birds.

![Figure 14. ZENK-ir cells counts in each brain region in response to tutor song for all three tutoring groups. Error bars are ± 1 SE](image)

Third, a possible, though improbable, explanation could be that the females in this experiment were not exposed to the different tutoring conditions prior to day 35 after hatching. Studies observing song acquisition processes of zebra finch have been described before and there is no evidence to suggest that zebra finches memorize songs before day 25 (Clayton, 1987; Immelman, 1969). In this experiment, females were not exposed to adult male songs post day 11 after hatching. Additionally, in other species, song memorization during fledging has not been found (Marler, 1970; Hultsch & Kopp, 1989). Thus, it is likely that the tutoring in my experiment coincided with a sensitive phase for song memorization.
In conclusion, my study does not provide evidence to support the forebrain auditory areas as the neural localization of the tutor song memory. However, it may support the hypothesis that ZENK activation in NCM and CMM reflect biological salience of the song playback, revealing attentional mechanisms, given that Zenk-ir was elevated in response to a variety of complex sounds in at least two brain regions.
References


Eales, L. (1987). Do zebra finch males that have been raised by another species still tend to select a conspecific song tutor? *Animal Behavior, 35*, 1347-1355.


Appendices

Abbreviations.

**Table 2. Table de Abbreviations.**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFP</td>
<td>anterior forebrain pathway</td>
</tr>
<tr>
<td>RA</td>
<td>robust nucleus of the arcopallium</td>
</tr>
<tr>
<td>DLM</td>
<td>dorsal lateral nucleus of the medial thalamus</td>
</tr>
<tr>
<td>LMAN</td>
<td>lateral magnocellular nucleus of the anterior nidopallium</td>
</tr>
<tr>
<td>Uva</td>
<td>nucleus uvaeformis</td>
</tr>
<tr>
<td>NIf</td>
<td>nucleus interfacialis of the nidopallium</td>
</tr>
<tr>
<td>PAm</td>
<td>nucleus parambigualis</td>
</tr>
<tr>
<td>MLd</td>
<td>mesencephaliculs lateralis pars dorsalis</td>
</tr>
<tr>
<td>Ov</td>
<td>nucleus ovoidalis</td>
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<tr>
<td>NCM</td>
<td>caudomedial nidopallium</td>
</tr>
<tr>
<td>NCMD</td>
<td>caudomedial nidopallium dorsal</td>
</tr>
<tr>
<td>NCMV</td>
<td>caudomedial nidopallium ventral</td>
</tr>
<tr>
<td>CMM</td>
<td>caudomedial mesopallium</td>
</tr>
<tr>
<td>CLM</td>
<td>caudolateral mesopallium</td>
</tr>
<tr>
<td>CST</td>
<td>caudal striatum</td>
</tr>
<tr>
<td>ZENK-ir</td>
<td>ZENK immunoreactive</td>
</tr>
<tr>
<td>IEG</td>
<td>immediate early gene</td>
</tr>
</tbody>
</table>
Subject: eSitus notification - New Animal Use Protocol is APPROVED 2007-089-08:5
Date: 22 September, 2011 9:21:25 PM EDT

Official Notice of Animal Use Subcommittee Approval

AUP Number: 2007-089-08
PI Name: Maciougall/shackleton, Scott A
AUP Title: Stress, Development And The Avian Brain

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Stress, Development And The Avian Brain"

* has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal 2007-089-08:5

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the AUS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care

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