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dissertation entitled

NEUROBIOLOGY OF SONG LEARNING AND PERCEPTION IN THE ZEBRA FINCH (*TAENIOPYGIA GUTTATA*), WITH A FOCUS ON THE ROLE OF THE HIPPOCAMPUS

presented by

David J. Bailey

has been accepted towards fulfillment
of the requirements for the

PhD degree in PSYCHOLOGY

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3-27-06
Date

NEUROBIOLOGY OF SONG LEARNING AND PERCEPTION IN THE ZEBRA FINCH (*TAENIOPYGIA GUTTATA*), WITH A FOCUS ON THE ROLE OF THE HIPPOCAMPUS

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Psychology

2006

ABSTRACT

NEUROBIOLOGY OF SONG LEARNING AND PERCEPTION IN THE ZEBRA FINCH (*TAENIOPYGIA GUTTATA*), WITH A FOCUS ON THE ROLE OF THE HIPPOCAMPUS

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Songbirds have become one of the most widely used model systems for the examination of neural processes underlying learning and memory. Zebra finch (*Taeniopygia guttata*) song is highly sexually dimorphic in that only males produce the behavior, yet perception of these vocalizations is critical to the reproductive success of both sexes. Males need to hear the vocalizations of a male tutor and, later, their own vocalizations in order to learn and develop song, but it is debatable whether experience plays a role in the development of song perception or preference in females. Vocal behavior in males is regulated by a system of interconnected nuclei that undergo dramatic morphological changes during development. However, other forebrain areas that ultimately receive input from the auditory thalamus show differential neuronal activity following conspecific or heterospecific song presentations in both sexes, suggesting that they are more important for the perception or perhaps neural storage of song. The hippocampus is also activated following playback of conspecific songs, suggesting that this area, traditionally associated with spatial memory, might play a role in song-related behavior. The present experiments began to test the role of early auditory experience in the development of female song perceptions, the development of neural responses to song in auditory regions and the hippocampus, the afferent connectivity of the hippocampal formation, and whether destruction of hippocampal tissue affects the consolidation or

retrieval of song templates or normal preferences for particular songs. These experiments show that (1) female zebra finches isolated from song but not biparental care during development respond like birds raised normally during conspecific song presentations but are initially limited in their ability to associate a novel environment with relevant song; (2) early in development, the sexes display different patterns of immediate early genes that may encode information about auditory stimuli but, over time, these responses become sexually monomorphic and likely remain that way in adulthood; (3) together with prior data indicating the efferent connectivity of the hippocampus, projections to the region indicate connections (some reciprocal) with nuclei involved in song behavior and suggest a role for the region in responses to vocal communications signals or auditory-related memories in the zebra finch as well as homology with the mammalian hippocampus; and (4) relatively small lesions of the hippocampus in males and females before the period song is learned, when it begins to be practiced (at least by males), or in adulthood have no effect on song preferences in males and females or song production in males, but do influence memory for a particular location. Taken together, these results indicate that the hippocampus, although not involved in song learning directly, may be integral in the consolidation of memories relating to song or the modulation of responses of other regions based on the salience of particular vocal signals or environments in which they are heard or produced.

To my wife, Amy, and my parents, Patricia and the late Lloyd Bailey

ACKNOWLEDGEMENTS

The research and writing of this dissertation would not have been possible without the support and encouragement of a number of individuals and departments within and outside of Michigan State University.

First, my gratitude to my advisor and mentor Juli Wade, for always being available to provide direction and assistance, and for graciously allowing me to pursue this particular area of research. To members of my dissertation committee: Laura Smale, who was a part of each of my committees since my first year at MSU, and Marc Breedlove, Tony Nunez, Lyn Clemens, and Steve Maren. I also thank Jane Witten and Fred Helmstetter of the University of Wisconsin-Milwaukee, who took me into their labs as an undergraduate and were instrumental in stimulating my interest in research in general and the study of biological psychology in particular. The collective efforts of all these individuals made me a more critical thinker and a better writer and researcher.

My thanks to countless graduate students, post-docs, technicians and undergraduates in the Wade lab who over the years provided help, friendship, and intellectual input and procedural support for my work, namely Erin O'Bryant, Camilla Peabody, Julia Rosebush, Stephanie Fuehring, Claudia Ruiz, Melissa Holmes, Katie Grausum, Matt Lovern, Sean Veney, Nancy Oberg, Casey Bartrem, Jenny Stynoski, Malik Williams, Lace Svec, Laurel Beck, Jen Neal, YuPing Tang, and Matt Burke.

Several individuals, like Shari Stockmeyer, made my day-to-day work more manageable, and were vital to particular experiments. David McFarlane was extremely helpful with computer, software and other technical support, as was Eric Weston with construction of the testing chambers.

I also express my extreme appreciation for funding from the National Institutes of Health, the Department of Psychology, the Graduate School, and the Council of Graduate Students.

I will always look fondly on my time in East Lansing thanks in large part to friends made there, like Russ Romeo, Dave Swender, Chris Wilson, Kalynn Schulz, Mike Schwartz, Mary Martin, Erich Ottem, and John Morris.

Thank you to my parents, Patricia and the late Lloyd Bailey, and my siblings, especially Thomas Bailey, for their steadfast support and encouragement, their continued interest in my work, and for keeping me focused on my career goals.

Finally, there is one person above all others to whom I owe thanks: my wife, Amy. She was and continues to be the foremost advocate of my career and a stabilizing force in my life. Her unwavering love, patience, and support, especially in the face of late nights and my occasional foul mood, was indispensable to surviving graduate school. Without her, in just about every way, this dissertation would not have been possible.

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KEY TO ABBREVIATIONS

ANOVA	analysis of variance
CA(1-3)	Cornu Amonnis (subfields 1-3)
CMM	caudomedial mesopallium
CON	conspecific song
DA	tractus dorso-arcopallialis
DLHP	dorsolateral subdivision of the hippocampus
DM	dorsomedial nucleus of the intercollicular complex
DLM	medial part of the dorsolateral thalamic nucleus
DMHP	dorsomedial subdivision of the hippocampus
DMP	posterior portion of the dorsomedial thalamus
DMSO	dimethylsulfoxide
dx	number of days post-hatching, with day of hatch as d1
HA	hyperpallium apicale
HD	hyperpallium densocellulare
HET	heterospecific song
HP	hippocampus
HVC	formal name for nucleus once referred to as "high vocal center"
IEG(s)	immediate early gene(s)
LAD	dorsal arcopallial lamina
LaM	mesopallial lamina
LFS	superior frontal lamina
LLV	ventral nucleus of the lateral lemniscus
lMAN	lateral portion of the magnocellular nucleus of the anterior nidopallium
mMAN	medial portion of the magnocellular nucleus of the anterior nidopallium
MSt	medial striatum
NCM	caudomedial nidopallium
NIf	nucleus interface of the nidopallium
NMDA	N-methyl-D-aspartate
NS	no song
nXIIts	tracheosyringeal portion of the hypoglossal nucleus
Ov	nucleus ovoidalis of the thalamus
PLSD	protected least significant difference
PVC	polyvinyl chloride
RA	robust nucleus of the arcopallium
SFL	superior frontal lamina
SPf	Substance P reactive nucleus
TON	randomly generated tones
UVa	nucleus uvaeformis
VHP	ventral subdivision of the hippocampus
X	Area X of the medial striatum
ZENK	Zif268, Egr-1, NGF1-A, Krox-24

CHAPTER ONE

In work first published in 1885, Herman Ebbinghaus detailed results from what is considered the first experimental investigation of learning and memory (Ebbinghaus, 1964), shifting away from studies of these psychological processes which were, at the time, introspective in nature. Using himself as the only subject, he demonstrated that associative functions could be examined experimentally and subjected to measurement. This work opened new avenues of learning and memory research that today continue to intrigue scientists, from a wide array of disciplines, who attempt to understand the modifications that occur within the nervous system that result in the maintenance and expression of behavioral change.

Much of what is known concerning the neural substrates of learning and memory has resulted from work with mammals, specifically rodents. Over the past few decades, how songbirds learn their songs, and the way in which they are able to discriminate between songs based on memories for them, has led to insights into these processes not possible with other model systems. Songbird research has provided, among other things, valuable insight into adult neurogenesis (Nottebohm, 2002b), a striking correlate to the development of human speech (Kuhl & Doupe, 1999), and a unique comparative model for the study of brain sexual differentiation (Wade, 1999). Of the songbirds, one of the most studied is the Australian zebra finch, *Taeniopygia guttata*. Zebra finches belong to the order Passeriformes, which are part of a limited group of vocal learners that also includes humans, cetaceans, parrots, hummingbirds (Jarvis, Ribeiro, da Silva, Ventura, Vielliard, & Mello, 2000) and perhaps elephants (Poole, Tyack, Stoeger-Horwath, & Waitwood, 2005). In their natural habitat, zebra finches are found living in social groups

in dry, wooded areas (Zann, 1996). In the wild, they breed year-round (although this depends on rainfall), and parents divide care of the young. They are also hearty breeders in captivity, making them an excellent model with which to study their behavior and its development.

Song Learning in Zebra Finches

A male zebra finch will sing to court a female, and females, who produce only structurally simple vocalizations, will choose a male to mate with based largely on his song quality (Zann, 1996). Males learn their songs during a defined stage in development when, from approximately post-hatch day 25 (d25) to d65, sons listen to their fathers' songs, memorize characteristics of them, and begin to rehearse their own (Eales, 1985; Immelmann, 1969; Nordeen & Nordeen, 1997). Beginning around d40 (Nordeen & Nordeen, 1997), males will rehearse the song template they have memorized, and through auditory feedback fine-tune it to closely match the song of their tutor (Brainard & Doupe, 2000; Nordeen & Nordeen, 1997). In general, many characteristics of adult male zebra finch songs are reflected in the songs of their male offspring (Böhner, 1983; Clayton, 1987). Isolation of zebra finch males prior to the sensitive period and through adulthood results in the production of an abnormal song (Eales, 1985; Immelmann, 1969). In fact, the number of song elements a juvenile copies from his father is directly proportional to the amount of time spent in contact with one another (Eales, 1985). When males are raised in the presence of a female alone, they begin to produce abnormal song that contains patterns characteristic of female calls (Eales, 1985). These males, however, can modify their songs by copying elements from males they encounter after d65 (Eales, 1985, 1987b), even copying over songs already learned (Eales, 1987b). The amount of

change to elements of song depends on the degree of social deprivation during development (Jones, ten Cate, & Slater, 1996). Also, male zebra finches, prevented from comparing their vocalizations to the memory of those from their tutors, can develop normal song when auditory feedback is reinstated close to maturity (Funabiki & Konishi, 2003). These results suggest that experience outside of the sensitive phase for song learning can produce behavioral change, and that the sensitive phase in males can remain "open-ended" if they have not learned song from a suitable tutor.

Female zebra finches clearly distinguish among songs that are relatively similar, but it is debatable whether they need to learn conspecific song during development to properly respond to it in adulthood. For example, females spend more time near a speaker broadcasting the song of their mate than the simultaneously presented song of another male (Miller, 1979a). They can also discriminate between the male songs of two subspecies, *Taeniopygia guttata guttata* and *Taeniopygia guttata castanotis* (Clayton & Pröve, 1989) and between their fathers' song and that of another male conspecific (Miller, 1979b; Riebel, Smallegange, Terpstra, & Bolhuis, 2001). Studies involving isolation from song during development suggest a potential sensitive period for learning in females as in males, and this may be dependent upon interaction or experience with their father or tutor. Females separated from their parents at d35 and presented with a simultaneous choice between their father's song and the song of another conspecific male show a preference for their father's song (Miller, 1979b). This preference is not detected in females acoustically isolated at d25 (Clayton, 1988). In addition, female zebra finches housed with only their mothers and tutored with taped male song show stronger and repeatable preferences for them than untutored females (Riebel, 2000). Cross-fostered

females of *Taeniopygia guttata guttata* and *Taeniopygia guttata castanotis* housed apart from their male siblings after d35 favor songs from males of the same subspecies as their foster-father, whereas females raised normally prefer the songs of males of their own subspecies (Clayton, 1990). Furthermore, male and female zebra finches isolated from song from d7 and raised by Bengalese finch foster mothers until d35 show a preference for hearing conspecific compared to heterospecific song when tested during their critical learning period (between d28 and d44; Braaten & Reynolds, 1999). Thus, while experience clearly guides the response of females to relatively subtle differences in song, it is not clear whether exposure to normal vocalizations during development is required for female zebra finches to recognize conspecific song as it is for males to produce it and when, if at all, a sensitive period for female song learning terminates (Riebel, 2003).

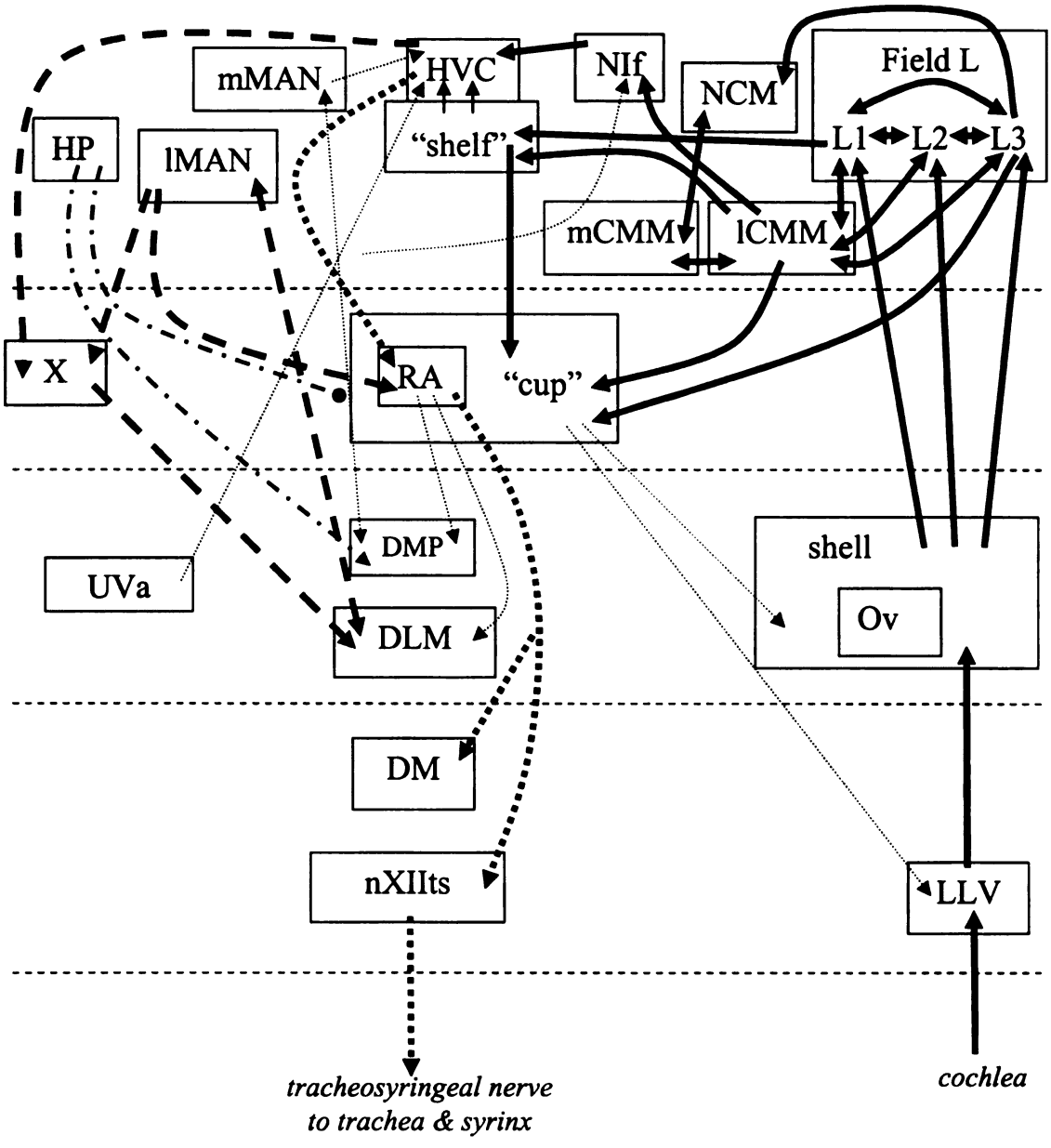
Song Control Regions and Circuits in Zebra Finches

Song behavior in males is regulated by a network of nuclei consisting of a pathway necessary for song learning interconnected with another important for song production (Figure 1.1). Recently, the nomenclature for avian telencephalic nuclei was revised (Reiner, Perkel, Bruce, Butler, Csillag, Kuenzel, Medina, Paxinos, Shimizu, Striedter, Wild, Ball, Durand, Güntürkün, Lee, Mello, Powers, White, Hough, Kubikova, Smulders, Wada, Dugas-Ford, Husband, Yamamoto, Yu, Siang, & Jarvis, 2004), and the new nomenclature is used throughout these chapters. Regions necessary for song acquisition, the lateral portion of the magnocellular nucleus of the anterior nidopallium (lMAN) and Area X of the medial striatum (MSt), are located in the anterior forebrain and show electrophysiological specificity for song that changes over development

(Doupe, 1997). Lesions of these nuclei during the sensitive period prevent normal song learning, while similar lesions in adult birds do not affect song (Bottjer, Miesner, & Arnold, 1984; Scharff & Nottebohm, 1991). Song production is regulated by HVC and the robust nucleus of the arcopallium (RA), and lesions to these nuclei at any point disrupt song output (Scharff & Nottebohm, 1991; Simpson & Vicario, 1990). In a dramatic example of structure mirroring function, these brain regions (with the possible exception of lMAN) are considerably larger in males who sing than females who do not (Nottebohm & Arnold, 1976).

In the past decade, the function of regions involved in the perception of song in zebra finches has been intensely investigated, made possible primarily by the study of gene products whose expression can be selectively induced in response to specific auditory signals (Clayton, 1997; Mello, 2002). Adult males that perceive their own song or other males' songs show upregulation of both the protein and message of two of these immediate early genes, c-FOS and/or ZENK (an acronym for Zif268, Egr-1, NGF1-A, Krox-24), in areas outside of the traditional song control nuclei described above. These regions are the caudomedial nidopallium (NCM) and caudomedial mesopallium (CMM) (Figure 1.1; Bolhuis, Hetebrij, den Boer-Visser, De Groot, & Zijlstra, 2001; Eda-Fujiwara, Satoh, Bolhuis, & Kimura, 2003; Jarvis & Nottebohm, 1997; Kruse, Stripling, & Clayton, 2000; Mello & Clayton, 1994; Mello, Nottebohm, & Clayton, 1995; Mello & Ribeiro, 1998; Mello, Vicario, & Clayton, 1992; Stripling, Kruse, & Clayton, 2001; Terpstra, Bolhuis, & den Boer-Visser, 2004). Conversely, when adult male zebra finches or canaries sing, neuronal activation is detected in the motor nuclei HVC and RA (Jarvis and Nottebohm, 1997; Kimpo and Doupe, 1997; Jin and Clayton, 1997; Mello and

Figure 1.1 Wiring diagram of structures in the zebra finch song circuit pulled together from tract tracing studies, detailing the high degree of known interconnectivities between the structures (Bottjer et al., 2000; Foster & Bottjer, 1998; Mello et al., 1998; Striedter & Vu, 1998; Székely, 1999; Székely & Krebs, 1996; Vates et al., 1996; Vates & Nottebohm, 1995; Vates et al., 1997). Solid lines specify auditory pathways, thick dotted lines primary motor pathways, and dashed lines song learning pathways. Thin dotted black lines designate portions of thalamo-cortical feedback loops, and the dashed + dotted lines indicate the only known connections of the zebra finch hippocampus with the regions indicated. DLM = medial part of the dorsolateral thalamic nucleus, DM = dorsomedial nucleus of the intercollicular complex, DMP = posterior portion of the dorsomedial nucleus of the thalamus, HP = hippocampus, lCMM = lateral portion of the caudomedial mesopallium, L1/L2/L3 = subdivisions of the Field L complex, LLV = ventral nucleus of the lateral lemniscus, lMAN = lateral portion of the magnocellular nucleus of the anterior nidopallium, mCMM = medial portion of the caudomedial mesopallium, mMAN = medial portion of the magnocellular nucleus of the anterior nidopallium, RA = robust nucleus of the arcopallium, NIf = nucleus interface of the nidopallium, nXIIts = tracheosyringeal portion of the hypoglossal nucleus, Ov = nucleus ovoidalis, UVa = nucleus uvaeformis, X = Area X of the medial striatum.


mMAN
HVC
NIf
NCM
Field L
L1
L2
L3
HP
lMAN
"shelf"
mCMM
lCMM
X
RA
"cup"
DMP
shell
Ov
UVa
DLM
DM
nXIIts
LLV
tracheosyringeal nerve
to trachea & syrinx
cochlea

Ribeiro, 1998). In NCM, heterospecific song presentations do not provoke such strong ZENK responses as conspecific song (Mello et al., 1992). In addition, significant positive correlations exist between the number of FOS- and ZENK-immunoreactive cells in the NCM of male zebra finches and the number of song elements copied from a tutor (Bolhuis et al., 2000; Bolhuis et al., 2001). The NCM of males therefore appears to be activated by specific acoustic signals that are particularly biologically-relevant.

Although females perceive subtle differences among vocalizations of male songbirds (see above), the areas of the brain responsible for this perception have not been intensely investigated. Areas traditionally assigned to male song behavior seem to play some role in song perception. For example, lesions of HVC in female canaries render them unable to distinguish conspecific from heterospecific song (Brenowitz, 1991) and to show a strong preference for conspecific song (Del Negro, Gahr, Leboucher, & Kreutzer, 1998). In contrast, in the zebra finch, lesions of HVC do not modify song preferences in females, but destruction of the neighboring CMM results in the display of similar responses to both heterospecific and conspecific song (MacDougall-Shackleton, Hulse, & Ball, 1998). Structures in the female songbird brain that show immediate early gene activation following song presentations have been investigated in a few studies. ZENK-immunoreactive cells in the NCM of female European starlings are significantly increased following exposure to song containing longer bouts; activation in the medial portion of CMM is uniformly high regardless of the length and number of song bouts presented (Gentner, Hulse, Duffy, & Ball, 2001a). Like males of this species, conspecific song presented to female European starlings results in an increase in the number of ZENK-immunoreactive cells in the NCM and CMM, expression that does not vary with

photoperiodic condition (Duffy, Bentley, & Ball, 1999). In female zebra finches, ZENK induction in the NCM following song presentation is qualitatively similar to that seen in males (Mello et al., 1992).

Work I have done (Bailey, Rosebush, & Wade, 2002) has provided a more detailed analysis of the neural response to song in adult female zebra finches. A higher density of FOS-immunoreactive nuclei is seen in the NCM of females following stimulation with conspecific compared to heterospecific song (Figure 1.2). This result parallels increased ZENK mRNA in the NCM of male zebra finches and canaries following conspecific versus heterospecific song presentations (see above). Interestingly, this pattern is also identified in the hippocampus (Figure 1.3). In CMM, FOS activation is equivalent following zebra finch and heterospecific song, yet both are increased compared to presentations of tone and silence (Figure 1.4). The CMM thus acts perhaps as an initial filter and the NCM likely provides a more detailed analysis of potential biological relevance. The function of the hippocampus in song behavior is unknown and warrants further attention.

Avian and Mammalian Hippocampal Function

The song-specific responses of a group of neurons within the hippocampus indicate a potential role for the structure in the processing of auditory information or song memories in zebra finches. The avian hippocampus appears to be homologous to the mammalian hippocampus based on numerous structural and functional factors and its role

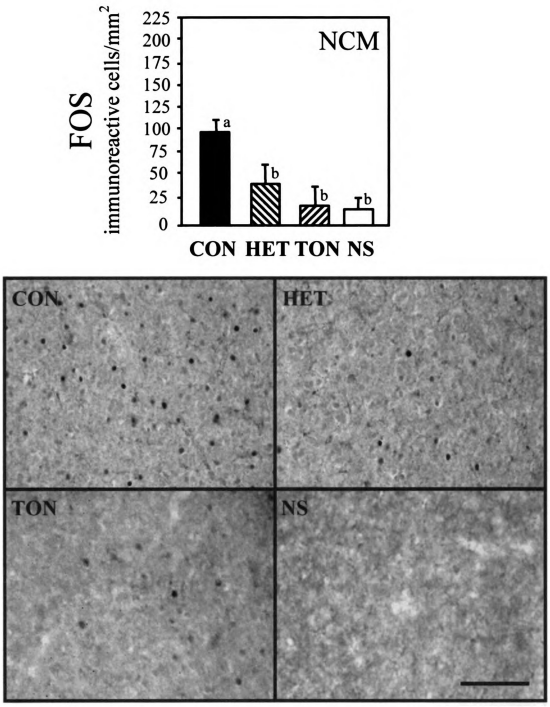


Figure 1.2. Density of FOS-immunoreactive cells (mean + SEM) in the NCM for adult females exposed to conspecific (CON) and heterospecific (HET) song, tones (TON) or no song (NS) (top panel). Different lowercase letters indicate significant differences between groups. The bottom panels contain photomicrographs of coronal sections through the zebra finch brain at the level of the NCM, showing FOS immunoreactivity in each of the stimulus conditions. Scale bar = 100 μm.

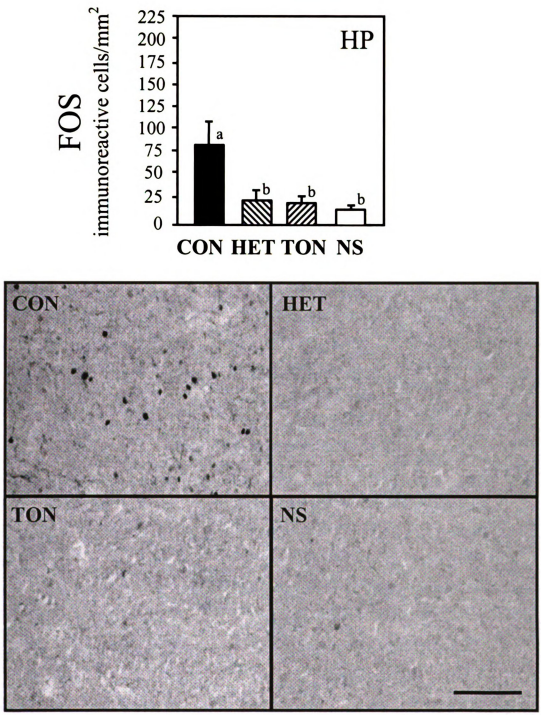


Figure 1.3. Density of FOS-immunoreactive cells (mean + SEM) in the hippocampus (HP) of female birds in the conspecific (CON), heterospecific (HET), tone (TON) and no song (NS) conditions (top panels). Different lowercase letters indicate significant differences between groups. Photomicrographs indicate FOS-immunoreactive cell nuclei for each group in the HP. Scale bar = 100 μm.

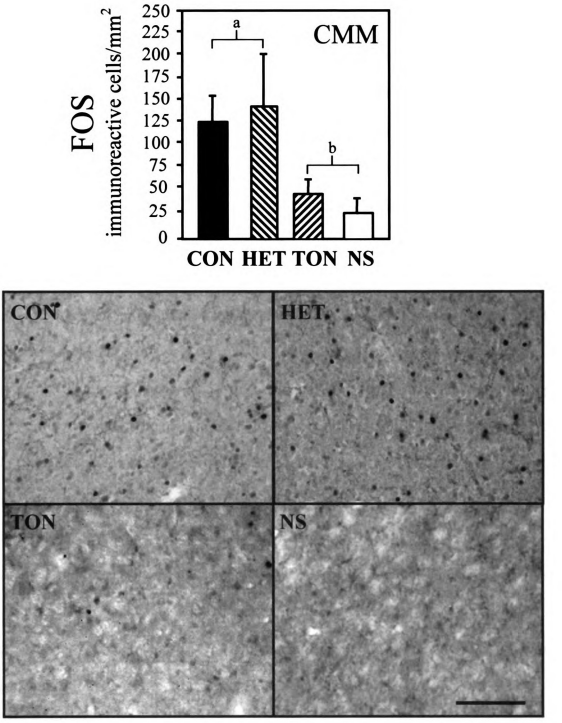


Figure 1.4. Density of FOS-immunoreactive cells (mean + SEM) in the CMM for adult females in the conspecific (CON), heterospecific (HET), tone (TON) and no song (NS) conditions. Different lowercase letters indicated significant differences between combined groups ("song" versus "no song"). Photomicrographs of sections at the level of the CMM showing FOS immunoreactivity in response to each of the stimulus conditions are shown in the bottom panel. Scale bar = 100 μm.

in spatial memory consolidation and retrieval (reviewed in Colombo & Broadbent, 2000; Macphail, 2002). The recovery of stored food is common in many avian species, specifically the parids and corvids (Kamil & Balda, 1990; Shettleworth, 1990). Remembering locations of food caches seems to be dependent on the hippocampus. For example, a bilateral lesion to the hippocampus in a black-capped chickadee reduces the accuracy of cache recovery but not the amount of caching nor attempts to find the stored food (Sherry & Vaccarino, 1989). Hippocampal volume in homing pigeons, which are able to navigate great distances back to their home lofts, is greater than that of a non-homing breed of pigeon (Rehkämper, Hasse, & Frahm, 1988). There are several exceptions, however, to the positive correlation between the size of the hippocampus and spatial memory ability (Bolhuis & Macphail, 2001). Lesions to the hippocampus produce deficits in recognition of the home loft and of surrounding familiar landmarks by homing pigeons; orientation back home is normal but slower (Bingman, Ioalé, Casini, & Bagnoli, 1988; Gagliardo, Ioalé, & Bingman, 1999; Strasser & Bingman, 1996).

In zebra finches, lesions of the hippocampus result in significant spatial memory impairment (Patel, Clayton, & Krebs, 1997a; Watanabe & Bischof, 2004), and transplantation of embryonic hippocampal tissue reverses this deficit (Patel et al., 1997a). Considering its importance in memory, the results I have obtained (Bailey et al., 2002) suggest that the hippocampus may somehow be involved in the formation or utilization of song memories or perhaps auditory perception. One possibility is that neuronal activation in the hippocampus may reflect consolidation of a "contextual song memory," the encoding of the environmental context in which biologically-relevant song is heard. Substantial hippocampal activity is not observed in animals that hear heterospecific songs,

tones or silence (Bailey et al., 2002; Bailey & Wade, 2003), which suggests that the hippocampus is not encoding information about a place specifically, but perhaps a relevant event that is occurring in that place. In the case of female zebra finches, this activity may result in an increase in preparedness to respond to male song in this context in the future. Although documented but not discussed in detail (Kimpo and Doupe, 1997; Bolhuis et al., 2000), activation in the hippocampus of males in response to song may reflect a comparable process that in this case results in future readiness to defend a nest site, for example. Although the hippocampus is a key component of the stress response (McEwen, 1999), it is unlikely that the immediate early gene activation observed was stress-related, such as that associated with removal from the aviary, handling by the experimenter, or exposure to a novel environment, since it would have been observed in the hippocampus in all birds. In addition, in rodents, c-FOS induction in the hippocampus is not influenced by the degree of a particular stressor but by the animal's ability to explore a novel environment, which amplifies the magnitude of spatial processing by the hippocampus and in turn the immediate early gene response (Pace, Gaylord, Topczewski, Girotti, Rubin, & Spencer, 2005).

This possible function of the zebra finch hippocampus - the integration of related events or stimuli in the environment - is consistent with a well-studied role of the hippocampus in mammals. The most dramatic and one of the earliest examples came from a report on, among others, a man named HM, whose temporal lobes were surgically removed to end his frequent epileptic seizures (Scoville & Milner, 1957). Following the bilateral lobectomy, HM was unable to recollect new people, places or things he encountered, and still today is unaware that he has become one of the most famous

subjects in the history of psychology. Shortly after the publication of HM's case, it was believed that the hippocampus was some sort of all-encompassing learning structure. It was later learned that the tissue surrounding the hippocampus in addition to the region itself that were removed from HM's brain likely caused the profound memory deficit (Squire, 1992), but there remains no doubt about the influence the hippocampus has on specific types of memory.

In the 1970s, the theory that the hippocampus may contain a "cognitive map (Tolman, 1948)" was put forward based on results that showed the activity of cells in the structure of freely moving rats was closely related to the animal's location in an open field (O'Keefe & Dostrovsky, 1971). Later, it was proposed that different sets of these "place cells" within the hippocampus represented a specific region of space (O'Keefe & Nadel, 1978). It is clear that the hippocampus contains cells responsive to an animal's place in its environment, and since the initial discovery a large number of studies have corroborated the original finding (Best & White, 1998; Eichenbaum, Dudchenko, Wood, Shapiro, & Tanila, 1999). However, contemporary theories of hippocampal function suggest that the structure may have a more general function in forming complex configural associations between various environmental stimuli, and these associations need not necessarily be spatial in nature. For example, the hippocampus is integral in the consolidation of the relationship between an unconditional stimulus (such as foot shock) and a novel environment (conditional stimulus; Kim & Fanselow, 1992; Phillips & LeDoux, 1995), the detection of novel stimuli (Knight, 1996), and the conveyance of an animal's location and what is currently present in that environment (Leutgeb, Leutgeb, Barnes, Moser, McNaughton, & Moser, 2005). It is now widely believed that the

hippocampus consolidates memories not only for places and events but also the temporal sequencing of those events (reviewed in Eichenbaum et al., 1999), processing which may (Clark & Squire, 1998) or may not (Chun & Phelps, 1999) require conscious awareness of cues and the context in which they are presented.

Comparison of Hippocampal Connectivity in Mammals and Birds

The mammalian hippocampus exhibits a set of three connected pathways known as the "trisynaptic" circuit (Amaral & Witter, 1989; Eichenbaum, Schoenbaum, Young, & Bunsey, 1996). In the first portion of this circuit, cells within the entorhinal cortex, which collect information from limbic association cortex and other association areas (Amaral & Witter, 1989; Eichenbaum et al., 1996), project to granule cells of the dentate gyrus via the perforant path. Second, cells of the dentate gyrus project to the large pyramidal cells of Cornu Amonnis or Ammon's horn, subfield 3 (CA3), via mossy fibers. Third, pyramidal cells in CA3 project to pyramidal cells of the CA1 subfield, via the Schaffer collateral system (Amaral & Witter, 1989). The output neurons of the hippocampus, primary pyramidal neurons of CA1, form a fiber bundle called the fornix that projects primarily to the mammillary bodies and septal nuclei (Amaral & Witter, 1989; Eichenbaum et al., 1996).

Intra- and interhippocampal connectivity in avian species may not be so different from that in mammals. In birds, the hippocampus (often cautiously referred to as the avian hippocampal complex or formation; Colombo & Broadbent, 2000) sits on the dorsal surface of the brain near the cerebellum. In the pigeon, sensory input from primarily the piriform cortex innervates the dorsolateral and dorsomedial subdivisions of

the hippocampus (Kahn, Hough II, Ten Eyck, & Bingman, 2003), the latter of which is subdivided into dorsal and ventral portions. Cells in the dorsal dorsomedial portion then project to ventrolateral, -central and -medial subdivisions, which project to the same subdivisions contralaterally and to cells of the ipsilateral ventral dorsomedial subdivision, whose axons exit the hippocampus. This network of connections in the pigeon hippocampus is comparable to the trisynaptic pathway in the mammalian hippocampus described above (Hough II, Pang, & Bingman, 2002).

The intratelencephalic connectivity of the homing pigeon hippocampus has recently been determined using anterograde and retrograde tracers (Atoji, Wild, Yamamoto, & Suzuki, 2002). Ipsilaterally, the hippocampus has reciprocal connections with the parahippocampal area, lateral septum, and nucleus taeniae (homologous to a portion of the mammalian amygdala; see below). Contralaterally, a unidirectional projection is observed from the hippocampus to the parahippocampal area, lateral septum, and MSt, and a reciprocal connection spans from the ventral portion of the hippocampus on one side and through the pallial commissure to the other.

Until recently, no clear anatomical lateral border between the avian hippocampus and surrounding tissue had been recognized (Kahn et al., 2003; Székely, 1999). A nucleus lateral to the hippocampus in pigeons has been identified (Erichsen, Bingman, & Krebs, 1991). Named SPf due to its high density of Substance P-immunoreactive neurons, its position relative to the hippocampus medially and the hyperpallium apicale (HA) laterally makes it a potential homologue to the mammalian entorhinal cortex (Kahn et al., 2003). Retrogradely labeled neurons within the zebra finch SPf were observed following injections of the anterograde tracer *Phaselous vulgaris* leucoagglutinin into the

dorsolateral hippocampus (Székely, 1999). In addition to these neurochemical markers, the lateral and anterior boundaries of the hippocampus can easily be visualized using a histochemical marker such as a Nissl stain, which reveal dramatic increases in cell density at the boundaries of the structure.

The efferent connections of the zebra finch hippocampal complex have been determined through injections of *P. vulgaris* (Székely & Krebs, 1996). The results of this study mirror the efferent projections and intraconnectivity of the hippocampal complex in pigeons (Atoji et al., 2002; Casini, Bingman, & Bagnoli, 1986; Krayniak & Siegel, 1978), the main difference being only three subregions were identified based on characteristic projections patterns: dorsolateral, dorsomedial, and ventral (Székely & Krebs, 1996). Tracer infusion into the dorsomedial subdivision indicated almost exclusively internal afferent projections. Terminating axons resulting from injections into the ventral region were found in the medial septum in both hemispheres and also in the contralateral hippocampus (Székely & Krebs, 1996). Dorsolateral injections of the tracer resulted in mainly ipsilateral projections, which terminated in the lateral hypothalamus, areas of the septum, nucleus taeniae, posterior portion of the dorsomedial thalamic nucleus, caudal arcopallium and the MSt. Innervation of the caudal arcopallium may cover areas such as the "cup" of RA that receives substantial descending auditory information from HVC (Mello et al., 1998). It is also interesting to note that the RA and hippocampus both send projections to the posterior portion of the dorsomedial thalamic nucleus (DMP; Vates et al., 1997; Székely & Krebs, 1996), an area that, together with traditional song motor control nuclei (HVC and RA), may coordinate the temporal organization of song (Vates et al., 1997). Nucleus taeniae has been shown, at least in Japanese quail, to be involved

in the control of sexual behavior (Absil, Braquenier, Balthazart, & Ball, 2002). In addition, the septum has been implicated in spatial memory and motivated behaviors in avian species (Shiflett, Gould, Smulders, & DeVoogd, 2002). Thus, connections exist that could allow the hippocampus to be involved in the processing of memories related to responses to song. However, afferents to the hippocampus must be determined to fully understand how the structure may interact with other regions known to play a role in song perception or possibly production.

Prospectus

The zebra finch provides an ideal model system for examining the development of neural mechanisms fundamental to multiple memory systems that control discrete behaviors. Relatively few studies have addressed the role of learning in the development of female song perceptions. To further examine this, the experiment in Chapter Two examines whether female zebra finches, which grew to adulthood without hearing their father's song throughout the majority of development yet remained in social contact with both parents, show a preference for conspecific versus heterospecific song. The remainder of the experiments tests the hypothesis that the hippocampus in both male and female zebra finches is important for the consolidation, retrieval or maintenance of song templates. Although much is known about the neural substrates of auditory perception in adult songbirds, less is known about its development. Specifically, if the hippocampus is important for the storage and/or retrieval of song memories, then the patterns or distribution of neuronal activity indicated by immediate early gene expression in the hippocampus and areas important for perception should increase or change during

development as song templates are acquired. The experiments in Chapters Three and Four were designed to identify the brain regions in juvenile zebra finches (at two separate and central developmental stages) that show immediate early gene activity in response to auditory stimulation, whether there are sex differences in that activation, and whether the immediate early genes examined are differentially expressed.

Further, if the hippocampus is important for the storage and/or retrieval of song memories, it should have connections with areas involved in song perception in both sexes and perhaps song production areas in males, and destruction of hippocampal tissue should impact song consolidation memories or perception as measured in adult males and females. Chapter Five details the afferent connectivity of the hippocampus in male and female zebra finches, including the areas with which it has reciprocal connections, specifically including the regions implicated in song learning, production or perception. Finally, Chapter Six evaluates the effects of hippocampal lesions in male and female zebra finches at the beginning of the song acquisition and sensorimotor integration periods (both as defined in males) and in adulthood, to determine the contribution of the structure to song learning (males and females), initial production and maintenance of learned song patterns (males), and spatial memory as a control (males and females). If the hippocampus is important in song learning, then males lesioned at the beginning of the template acquisition period should produce abnormal song as adults. Decrements in adult song production should be observed if the hippocampus is integral in the auditory feedback necessary for the fine-tuning of song that begins at sensorimotor integration. If the structure is important for maintenance of the song template, then lesions of the hippocampus in adulthood should result in abnormal song. These possibilities are not

mutually exclusive. Given the song-specific induction of immediate early genes I observed in the hippocampus (Bailey et al., 2002), lesions of the structure may result in an inability to properly respond to conspecific songs in adulthood. If the hippocampus is important in song learning, then lesions during development should impact consolidation of a song template and thus influence song preference in adulthood. As mentioned above, if the region is important in maintenance of a song template, then lesions in adulthood should result in abnormal song preferences. This last experiment only begins to test the importance of the hippocampus in auditory learning in zebra finches; it does not test whether activity within the structure is due to consolidation of a "contextual song memory," which links a relevant auditory stimulus with a novel or familiar environment.

CHAPTER TWO

Song exposure during development modifies adult behavior following song perception in the female zebra finch

INTRODUCTION

The development of responses by female birds to the songs of males has been described as a "neglected" but important area of study in bird song research and vocal communication in general (Slater, 2003). It is clear that female zebra finches (*Taeniopygia guttata*) can distinguish among relatively similar songs. These birds, who produce only structurally simple vocalizations, spend more time near the songs of their mates than the simultaneously presented song of other conspecific males (Miller, 1979a). They can discriminate between the male songs of two subspecies, *Taeniopygia guttata guttata* and *Taeniopygia guttata castanotis* (Clayton & Pröve, 1989), and between their fathers' songs and those of other zebra finch males (Miller, 1979b; Riebel, Smallegange, Terpstra, & Bolhuis, 2002). But, females may not detect specific features of zebra finch song as males do. They require more trials than males to distinguish between songs of males from their own aviary (Cynx & Nottebohm, 1992), as well as songs played in reverse (Cynx, 1993), and their preference for their fathers' songs does not generalize to the songs of male siblings, which share characteristics of the fathers' songs (Riebel & Smallegange, 2003).

Juvenile exposure to song is critical for normal masculine song development. Isolation from song as juveniles dramatically alters the structure of adult vocalizations produced by many species of songbirds (Marler, 1997). In zebra finches, song learning occurs during a defined stage in development when, from approximately post-hatch day 25 (d25) to d65, sons listen to their fathers' songs, memorize characteristics of them, and

begin to rehearse their own (Eales, 1985; Immelmann, 1969; Nordeen & Nordeen, 1997). In general, many characteristics of adult male zebra finch songs are reflected in those of their male offspring (Böhner, 1983; Clayton, 1987). Isolation of zebra finch males prior to the sensitive period and through adulthood results in the production of an abnormal song, which is "slow" and contains patterns uncharacteristic of normal adult zebra finch song (Eales, 1985; Immelmann, 1969). Indeed, the number of song elements a juvenile copies from his father is proportional to the amount of time spent in contact with one another (Eales, 1985). When males are raised in the presence of a female alone, they begin to produce abnormal song that contains patterns characteristic of female calls (Eales, 1985). These males, however, can modify their songs by copying elements from males they encounter after d65 (Eales, 1985, 1987b), and the amount of change to elements of song depends on the degree of social deprivation during development (Jones et al., 1996). These studies suggest that experience outside of the sensitive phase for song learning can produce behavioral change, and that this period can remain "open-ended" to some extent if song was not learned from a suitable tutor. Recent work also shows that male zebra finches, prevented from comparing their vocalizations to the memory of those from their tutor(s), can develop normal song when auditory feedback is reinstated close to maturity (Funabiki & Konishi, 2003).

The early stages of song learning (template formation beginning around d25) may be similar in the two sexes (for review, see Riebel, 2003). Females separated from their parents at d35 and as adults presented with a simultaneous choice between their father's song and the song of another conspecific male show a preference for their father's song (Miller, 1979b). This preference is not detected in females acoustically isolated at d25

(Clayton, 1988), suggesting that aspects of song learning may occur from d25 to d35. Similar results are obtained when preference for "super-normal length" zebra finch song is measured. Female zebra finches separated from their parents at d25 show an equal amount of time near normal and super-length song, whereas separation at d35 (or d70) results in the normal adult preference for the longer songs (Neubauer, 1999). Additionally, females housed with only their mothers and tutored with taped male song show stronger and more repeatable preferences for them than untutored females (Riebel, 2000).

While experience shapes the responses of females to relatively subtle differences in song, it is not clear whether exposure to normal vocalizations during development is required for adult female zebra finches to recognize conspecific song as it is for males to produce it. A recent study documented that female zebra finches spend more time in the side of a chamber near conspecific compared to canary song, regardless of whether they were raised with males (Lauay, Gerlach, Adkins-Regan, & DeVoogd, 2004). This result is consistent with the existence of an inherent preference for at least some characteristics of zebra finch song, although other possibilities exist. The lack of preference in females raised without adult males does not appear to be influenced by the songs of male siblings, suggesting either that templates may be formed by females during a sensitive period terminating before their brothers begin to vocalize (around d40) or that untutored male song is insufficient to stimulate development of this female characteristic. In a similar study, both male and female zebra finches isolated from song from d7 and raised by foster mothers until d35 showed a preference for hearing conspecific compared to starling song as juveniles (Braaten & Reynolds, 1999). In both of these studies, however, the

responses were compared between zebra finch song and that of only one other species. Thus, it is not clear whether the preference would broadly apply. In addition, the effects of adult experience were not taken into account. Braaten and Reynolds (1999) did not test mature animals, so it is unclear whether the preference they discovered would be maintained at a time when females choose mates. In Lauay et al. (2004), the test for conspecific versus heterospecific song was conducted after the females (even those who developed without song exposure) heard some song as adults. Their behavior may therefore have been influenced by recent exposure.

While this general question (whether female zebra finches need to learn the song of their species to respond selectively to it in adulthood) has been investigated twice, the present study was designed with methodological differences as follows. To address the points above, adult responses to several songs of each type (zebra finch and heterospecific) were examined alternately over the testing days in order to determine whether responses would generalize (Kroodsma, Byers, Goodale, Johnson, & Liu, 2001; Kroodsma, 1989). In addition, since zebra finches are extremely social (Zann, 1996), and prohibiting contact with parents and siblings during development impacts auditory discrimination capability in adulthood (Sturdy, Phillmore, Sartor, & Weisman, 2001), females were kept in social contact with their fathers through what is typically considered the sensitive period for template formation (see above). Rather than removing fathers, the tracheosyringeal nerves were severed so they could not sing. Finally, immediate early gene activation is increased in the hippocampus as well as auditory areas in adult and juvenile female zebra finches following conspecific song exposure (Bailey et al., 2002; Bailey & Wade, 2003). This result is consistent with the idea that females encode

information about where a favorable song was heard. Therefore, in addition to testing responses during song, the "contextual" memory of these birds was examined by observing their responses to the environment in which conspecific and heterospecific song was heard the day before.

MATERIALS AND METHODS

Animals and Housing

All zebra finches were obtained from the breeding colony at Michigan State University. Prior to group assignment, birds were housed in communal aviaries consisting of approximately seven breeding pairs and their young. Nest boxes in the aviaries and used in the testing chamber were constructed of polyvinyl chloride (PVC), and were 12.7 cm high and 12.7 cm in diameter with a 3.8 cm diameter hole 2.5 cm from the top. A 5 cm long perch extended outward below this hole, and the top of each nest box was covered with a removable black Plexiglas disc. Seed and water were provided *ad libitum*, and supplements of spinach or oranges and a mixture of hard-boiled chicken eggs and bread were provided weekly. The light:dark cycle was 12:12 (lights on at 0700).

Experimental Manipulation

As eggs hatched in nest boxes in the main bird colony, the mother and father were determined by daily observation until the identity of the male and female that consistently entered and spent time in each nest box could be confirmed. Two to four days before a nest box was to be moved (see below), fathers were temporarily removed and their song recorded in the presence of a novel female. An axotomy or sham surgery was then

performed depending on whether the nest box was to be moved to the song isolation or control condition, respectively. Each father was anesthetized with Metofane, and an incision was made in the skin above the trachea. For males entering the song isolation condition, the tracheosyringeal portions of the hypoglossal nerves (XIIts) were gently separated from the trachea and 4-6 mm was removed bilaterally. Fathers entering the control condition underwent a sham surgery in which the nerves were gently separated from the trachea but not severed. The axotomy produced complete cessation of normal song production, and resulted in sounds associated only with respiration (Figure 2.1). Following recovery from surgery, each animal was returned to its aviary. All fathers of experimental females in this study showed no apparent difficulty in breathing, or any signs of poor health, and all quickly resumed care of their young.

After all of the viable eggs hatched and before the first hatchling reached d8, the nest box and parents were moved to the control condition (to a separate aviary in the main bird room with similar groups; n = 11 females from 3 different clutches) or to the song isolation condition (to a separate room that housed only females, axotomized fathers and their offspring; n = 10 females from 4 clutches). Around d40, when the plumage of zebra finches was developed enough to allow distinction between males and females, males raised in the song isolation condition were removed before they began to sing (Immelmann, 1969; Nordeen & Nordeen, 1997). At this time both parents were also removed, as were male siblings and parents of control animals. Each father in the song isolation condition was given an overdose of Equithesin following the recording of vocalizations in the presence of a female. None produced vocalizations resembling song

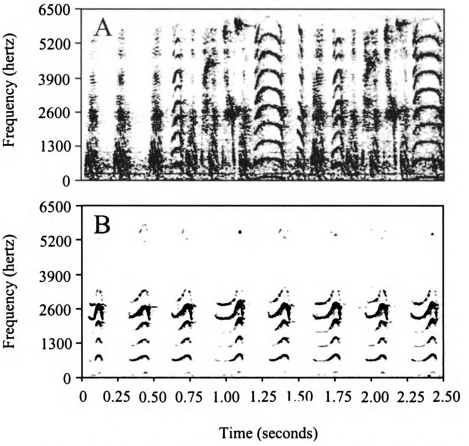


Figure 2.1. Sonagram of vocal behavior in the presence of a novel female before (A) and after (B) severing of the tracheosyringeal nerves of a father of birds in the song-isolated group. While rhythmic tones associated with respiration were consistently heard, song was never detected following axotomy.

(Figure 2.1). At this time, the tracheosyringeal nerves of axotomized fathers were checked. An average of at least 3-4 mm remained between the severed ends of each nerve; in no case did they regenerate enough to make contact with one another.

Behavioral Testing

The testing apparatus (Figure 2.2), located in a quiet room away from the main bird colony, was constructed of a wood frame, steel mesh sides, back and top, and a Plexiglas front through which observations and video tapes were made. Sound attenuating foam covered the walls surrounding the entire apparatus. Two sets of three perches were located at each end of the chamber, and a single perch was in the middle. Nest boxes were located at each end of the chamber. Hidden in each nest box were speakers that delivered song.

Female-directed zebra finch songs were recorded from males from our colony. Heterospecific songs were obtained from The National Geographic Society and Cornell Laboratory of Ornithology's *Guide to Western Bird Songs*. Songs from six different species (Bell's vireo, Cassin's finch, marsh wren, American robin, summer tanager, white-breasted nuthatch) were recorded from this guide and were chosen based on their overlapping frequency and similarity in bout length to zebra finch song. Speakers, attached to a PC, delivered song stimuli at 72 dB at a distance of 12 cm from each. Real Jukebox (version 1.0.0.488) controlled song delivery. Auditory responses to song presentations were recorded using a portable cassette recorder (Marantz, Aurora, IL) connected to an Optimus boundary microphone (Radio Shack), and a video camera

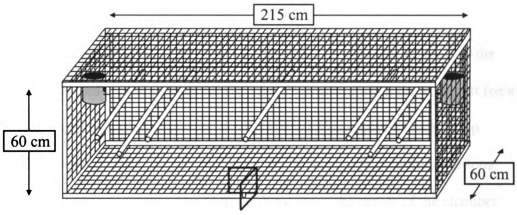


Figure 2.2. Chamber used for song presentations. The apparatus was constructed of a wood frame, steel mesh sides, back, and top, and a Plexiglas front through which observations and video recordings were made. Two sets of three wooden perches were located at each end of the chamber, and a single perch was in the middle. The middle perches in the sets of three were located 20.2 cm above the floor of the chamber, and were spaced 6.3 cm from the adjacent perches. All other perches were 30 cm above the floor of the chamber. Nest boxes were located at each end of the chamber. Nest boxes were constructed of polyvinyl chloride (PVC), and were 12.7 cm high and 12.7 cm in diameter with a 3.8 cm diameter hole 2.5 cm from the top. A 5 cm long perch extended outward at 90° below this hole, and the top of each nest box was covered with a removable Plexiglas disc. Hidden in each nest box were speakers that delivered song, and sound attenuating foam covered the walls surrounding the entire apparatus.

recorded movements. Fluorescent room lights were turned off during testing, and 60 watt incandescent light bulbs in metal reflectors located 30.5 cm above each end of the chamber were illuminated. All testing was done during the light portion of the light:dark cycle.

To ensure similarity in recent history between the two groups, animals in the control condition were moved in with the song isolation group when the youngest female in each clutch was d80. As adults (mean = d121.2 +/- 2.3), females in each group underwent behavioral testing. At the beginning of a session, a female from the song isolation or control condition was placed through a door in the center of the chamber described above, facing the back wall. Following a 3 minute acclimation period, conspecific or heterospecific song was randomly delivered as follows. Each session (one a day for twelve days) consisted of one conspecific or heterospecific song played from the right or left speaker. All song files were 30 seconds in length (to maximize consistency across song exposures) and were repeated six times, for a total presentation time of 3 minutes. Over the course of the testing sessions, each female was exposed to all six conspecific and six heterospecific songs (Kroodsma et al., 2001; Kroodsma, 1989), with the order randomly chosen before each session. Whether conspecific or heterospecific song was played on the first testing day was counterbalanced, as was initial side of delivery (right or left speaker). Birds heard each of the twelve songs once, and conspecific and heterospecific song were alternated on testing days. Three minutes after song presentations, birds were removed from the testing apparatus and were immediately returned to the group aviary.

Data Analysis

Birds primarily responded with calls and movements; few other behaviors were observed. Long and short calls were tallied (see Zann, 1996 for a description of call types). If a bird turned 180° to face the opposite direction, this was scored as a "turn." "Perch jumps" were defined as movement from one perch to another, a flight from a perch and a return to that same perch, or a flight from a perch to the top of a nest box or vice versa. As indicated above, the data were analyzed as responses during, immediately after, and 24 hours after zebra finch and heterospecific song presentations. Since conspecific and heterospecific song delivery was counterbalanced, some birds were presented with zebra finch song on testing day 1 and others first heard it on testing day 2. Thus, while all birds were presented with six songs of each type, data from only one-half of the animals in each group were available for the day after the sixth zebra finch or heterospecific song presentation, so those data were discarded.

Data were scored live, as well as from video and audiotapes by an observer not aware of treatment conditions. All data reported are from the tapes, except for that from four females for one testing session in which technical difficulties precluded electronic data collection. Tests for skewness and kurtosis revealed the data were not normally distributed, so square root transformations were performed. All statistical analyses were carried out on the transformed data, although the means and standard errors depicted are derived from the raw data. Effects of developmental condition (control versus song isolation; between individual birds), stimulus type (conspecific versus heterospecific song; within individuals), time (across five tests; within individuals) and period (during, immediately after, or 24 hours after song; within individual birds) were assessed using

analyses of variance (ANOVA). When significant effects were detected, pairwise planned comparisons were used (Maxwell, 1980). Statistical analyses were performed using Statview and SAS (both SAS Institute).

RESULTS

The statistical significance of effects reported for the overall ANOVA were similar regardless of whether the number of calls or the amount of movement (turns and perch jumps), or the sum of all of these behaviors was used, except in three instances. These differences are indicated below, but otherwise for simplicity, data are reported only for the total of calls plus movements.

During the 3 minute acclimation period before their first song exposure, the number of calls and movements by each group was equivalent and relatively low, although a trend existed for song-isolated birds to be more active ($t(19) = 1.92$, $p = 0.070$; Figure 2.3). The quantity of responses differed dramatically across the periods in which they were assessed (while song was playing, immediately after song, or 24 hours after; $F(2, 38) = 89.15$, $p < 0.0001$; Figure 2.4). Calls and movements during song (Figure 2.4A) were two times greater than those immediately after (Figure 2.4B; $t(20) = 4.33$, $p = 0.0003$). Interestingly, responses 24 hours after song presentation were approximately four times more than those during ($t(20) = 7.68$, $p < 0.0001$) and 8 times those immediately after ($t(20) = 12.74$, $p < 0.0001$) song (Figure 2.4C). Across the 5 analyzed tests, responses also differed ($F(4, 76) = 5.39$, $p = 0.0007$), and this variable of time interacted with period when the sum of the behaviors were considered (total responses:

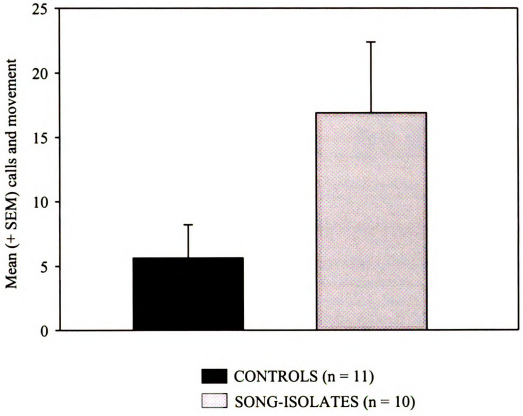
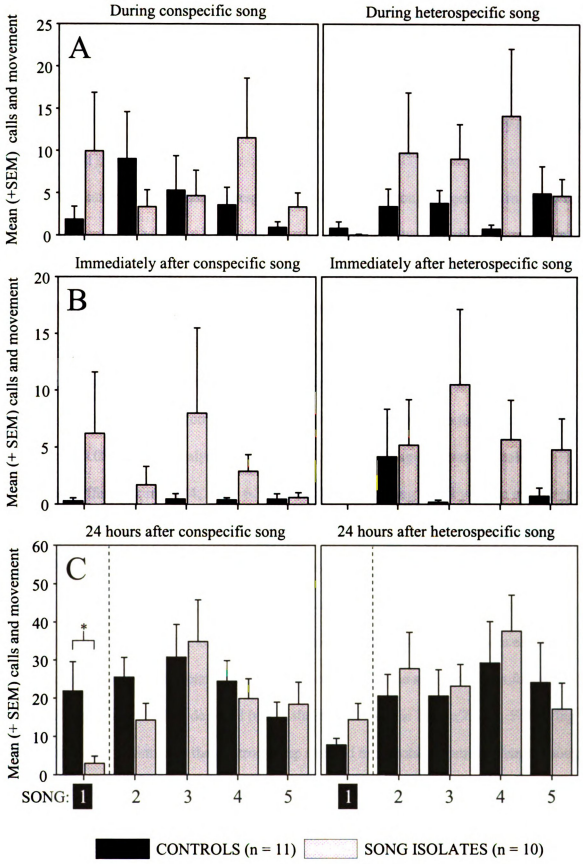


Figure 2.3. Mean (+ SEM) calls plus movement before song exposure on the first testing day. These baseline values were not significantly different, although the song isolates appeared somewhat more active.

Figure 2.4. Mean (+ SEM) calls plus movement during five conspecific and heterospecific song presentations (A), immediately after those songs (B), and 24 hours after (C) by females raised with exposure to zebra finch song (CONTROLS) or isolated from song early in life through adulthood (SONG ISOLATES). Responses 24 hours following the first conspecific and heterospecific songs are highlighted by dashed lines. The "*" indicates a significant difference between the groups. Note differences in the Y-axis scales.


During conspecific song
During heterospecific song
A
Mean (+SEM) calls and movement
25
20
15
10
5
0
Immediately after conspecific song
Immediately after heterospecific song
B
Mean (+ SEM) calls and movement
20
15
10
5
0
24 hours after conspecific song
24 hours after heterospecific song
C
Mean (+ SEM) calls and movement
60
50
40
30
20
10
0
*
SONG: 1 2 3 4 5 1 2 3 4 5
CONTROLS (n = 11)
SONG ISOLATES (n = 10)

$F(8, 152) = 2.16$, $p = 0.033$; calls only: $F(8, 152) = 1.46$, $p = 0.178$; movement only: $F(8, 152) = 1.19$, $p = 0.311$). Main effects of group (developmental condition) and song type (conspecific versus heterospecific song playbacks) were not detected, and none of the two, three, or four-way interactions involving those variables were statistically significant (all $p \geq 0.171$).

The substantially enhanced responses 24 hours after song suggested a memory for the testing experience the day before and warranted further attention. For control birds, this value after the first zebra finch song was more than four times greater than that during the acclimation period in the first test (before they were exposed to any songs in adulthood). As it was in the silent periods following song exposure in each test, behavior of the song isolates was somewhat higher and considerably more variable, but it did not differ significantly from the control birds during that first exposure to the chamber ($t(19) = 1.82$, $p = 0.0842$). The analysis of values 24 hours after song exposure revealed a significant effect of time ($F(4, 76) = 6.30$, $p = 0.0002$) and song type by group interaction ($F(1, 19) = 4.87$, $p = 0.0399$; calls only: $F(1, 19) = 3.15$, $p = 0.0920$). This significant effect of time appeared largely due to differences in responses of the controls and song isolates during the early testing sessions. Thus, responses of the birds to the chamber in which they heard conspecific or heterospecific song for the first time 24 hours earlier were analyzed, and a significant interaction between song type and group was found ($F(1, 19) = 6.47$, $p = 0.0198$; data depicted before the dashed lines in Figure 2.4C). Following zebra finch song, animals in the control group showed more total responses than the song isolation group ($t(19) = 2.74$, $p = 0.0129$; Figure 2.4C, left). No significant difference was found between the two groups in the number of total responses during the same

period 24 hours following heterospecific song (t (19) = 0.73, p = 0.470; Figure 2.4C, right).

DISCUSSION

Adult female zebra finches responded considerably more during than immediately after song. Thus, auditory stimuli modified behavior in both the song-isolated and control groups. The behavior was further increased when females were re-introduced into the testing chamber 24 hours following conspecific or heterospecific song presentations. This enhanced delayed response suggests a memory for the experience the day before. Intriguingly, females isolated from song throughout most of their development initially showed fewer responses in the chamber in which zebra finch song was heard 24 hours earlier than females that were raised in the presence of singing males. This result is consistent with the idea that females differentially form representations of environments in which song was heard depending on their prior exposure to song. Over time, however, differences between the groups were eliminated. The average number of calls and movements by the group raised without song was equivalent to the control birds by their third exposure to zebra finch song, which suggests that adult experience can fairly rapidly overcome deficits in responses due to developmental isolation from song. In both groups, these responses remained consistently high over the testing sessions (did not habituate across tests) likely due to the fact that birds never heard the same conspecific or heterospecific song more than once.

At least three explanations exist for why females raised in isolation from zebra finch song do not initially respond to the environment in which it was heard to the same

degree as controls. First, it is possible that responses by song-isolated females were limited due to increased attention in anticipation of song stimuli presentations that they experienced or learned for the first time 24 hours earlier. In experiments using song playback, a bird orienting to the source of a song while remaining stationary is typical, and may be as sensitive a measure as copulation solicitation displays (Searcy, 1992). Second, without prior experience with conspecific song, the stimulus may not have been salient enough to create an association with the environment. Third, this deficit could be the result of cue competition as seen in studies of classical conditioning (Wasserman & Miller, 1997). Females familiar with conspecific song appear capable of associating it with where it was heard, resulting in a "conditional response" to the chamber 24 hours later (calls and movement in anticipation of song presentation). In song isolates, zebra finch song was novel and therefore may have competed with the environment for being encoded in memory, resulting in an attenuation of conditional responding one day later. Given that responses by the two groups 24 hours after song became equal over time, the associative strength of the stimuli likely changed as a result of experience with song by those birds previously isolated from it.

This ability of adult females raised without hearing song to "catch up" in their response to the context in which song was heard after only a few exposures is consistent with some other studies showing that song-related learning can occur later than the d25 to d35 "critical" period (see Introduction; Clayton, 1988; Miller, 1979b; Neubauer, 1999; Lauay et al., 2004). Female and male zebra finches can learn at least some features of the songs of conspecifics as late as four to six months post-hatching (Clayton, 1988). Additionally, like the song-isolated females in the present experiment, song features can

be learned or modified by males at a later age than normal (even at sexual maturity) if they are deprived of an appropriate tutor (Clayton, 1987; Eales, 1985, 1987a; Jones et al., 1996) or auditory feedback (Funabiki & Konishi, 2003) during the typical ages for song acquisition. Also similar to females in the present experiment, adult males can form memories of song following relatively little (3 hours) exposure to it (Stripling, Milewski, Kruse, & Clayton, 2003). Thus, males and females seem capable of learning some discriminations rather quickly, and in females this can occur even after isolation from song during development.

It is possible that some innate knowledge of the general features of song exists in zebra finches (Braaten & Reynolds, 1999), which facilitated the observed changes in adult behavior based on relatively little exposure. However, it is also possible that the developmental experience of even the song-isolated birds had some impact. That is, birds in both groups were exposed to females who may have produced calls. These calls have some characteristics of male song, such as harmonic stacks (Vicario, Navqi, & Raksin, 2001; Zann, 1996), which may have been sufficient to induce some later zebra finch song recognition. Similarly, three of the heterospecific songs (Bell's vireo, marsh wren, and white-breasted nuthatch) contained harmonic stacks and along with the exposures to zebra finch songs may have also contributed to development of the song-context association during the early trials. These results are similar to those in female canaries, who recognize conspecific song when reared without exposure to it, but acoustic tutoring influences the development of song preferences (Nagle & Kreutzer, 1997).

The present data also suggest that female zebra finches appear able to develop the association between conspecific song and its environment using the features of song from any normal, adult male, since the songs on each of the testing days were randomly selected from a bank of six. This idea is similar to what can occur in males. While they typically learn from their fathers or males whose songs resemble their fathers' (Böhner, 1983; Clayton, 1987; Immelmann, 1969), juvenile males can readily learn from other available tutors, and do not require physical contact, social attachment, or even a live bird to do so (Bolhuis, Van Mil, & Houx, 1999; Eales, 1985; Houx & ten Cate, 1999; Mann & Slater, 1994; Williams, 1990).

The neural mechanisms involved in the formation of a memory for conspecific song, including the environment in which it is heard, are currently unclear. However, in mammals and birds, the hippocampus mediates the consolidation of the association of events in the environment (Colombo & Broadbent, 2000; Eichenbaum, 2000; Macphail, 2002). Birds possess a wide range of spatial/contextual memory abilities, that at least in part depend on the hippocampus, which is similar structurally and functionally to its mammalian homologue (Colombo & Broadbent, 2000; Macphail, 2002; Székely, 1999; Székely & Krebs, 1996). Lesions of a portion of the hippocampus in male zebra finches result in significant spatial memory impairment (Patel et al., 1997a; Watanabe & Bischof, 2004), a deficit reduced by transplantation of embryonic hippocampal tissue (Patel et al., 1997a). Additionally, immediate early gene activation is observed in the hippocampus of adult and juvenile female zebra finches following exposure to zebra finch song compared to other auditory stimuli (Bailey et al., 2002; Bailey & Wade, 2003), suggesting a potential relationship between spatial or contextual memory and conspecific song

exposure. Isolation from song throughout development may initially impact the ability of the hippocampus and/or other regions in the female zebra finch brain to accurately detect and/or respond to conspecific song and associated stimuli. Future work will determine the potential involvement of the hippocampus in the consolidation of memories related to song.

As indicated in the Introduction, this potential involvement of the hippocampus in song-related memory was an important consideration in the analysis of this experiment. It also influenced the decision about whether to treat the females with estradiol. The hormone tends to increase responsiveness in experiments examining female preference for male song (see Searcy, 1992). However, the hippocampus of adult zebra finches synthesizes estradiol and contains neurons that express estrogen receptors (Gahr, Güttinger, & Kroodsma, 1993; Saldanha, Clayton, & Schlinger, 1999). Estradiol upregulates NMDA receptors and thus likely influences neuroplasticity in the zebra finch hippocampus (Saldanha, Clayton, & Schlinger, 1999; Saldanha, Schlinger, Micevych, & Horvath, 2004). These issues, combined with the noted effects of estrogen on memory in other vertebrates (McEwen & Alves, 1999), and the possible contribution of the hippocampus to song-related memories in the zebra finch (see above), suggested that estradiol could have confounded the results of the present study, so the levels were not artificially increased. Similarly, the moving of control females in with song-isolated birds before testing was an attempt to equalize recent auditory history, thus focusing on differences between the groups due to their auditory experiences only during development. However, exposure to conspecific (and, surprisingly, heterospecific) song enhances follicular development over no song playback in female canaries (*Serinus*

canaria; Bentley, Wingfield, Morton, & Ball, 2000). If zebra finches are similar in this regard, then the lack of recent exposure to males may have further lowered their circulating estradiol concentrations. This deficit in estradiol may explain why the typical overall preference for zebra finch song was not detected, and why behaviors such as copulation solicitation displays were not seen. This idea could readily be tested in future studies.

Unlike other studies (Clayton, 1988; Lauay et al., 2004; Lauay, Gerlach, Adkins-Regan, & DeVoogd, in press; Miller, 1979b), birds in the present study did not spend more time near individual ends of the chamber that broadcast the song stimuli, likely because songs played from the two speakers could be heard from anywhere in the apparatus. It is also possible that other methodological differences between the present and previous studies prevented our females from spending more time near zebra finch than heterospecific songs. For example, perhaps females needed visual stimuli to encourage responses, such as models of male zebra finches (Lauay et al., 2004). Without this measure of preference, it may be difficult to know precisely what the increases in behavioral activity by females mean (for example, whether they have a positive or negative connotation). However, calls (which in the present study contributed substantially to the effects detected) may indicate song preference; data from canaries suggest that this response to playbacks is similar to that of copulation solicitation displays (Nagle, Kreutzer, & Vallet, 2002).

In any case, several points can be gleaned from this experiment on female zebra finches. First, they clearly form memories for experiences involving auditory stimulation that are maintained for at least 24 hours. Second, developmental exposure to conspecific

song initially enhances the response in the day following this experience, indicating that the memory (song-context association) is stronger or that its potential consequences are more salient in females who had prior experience with zebra finch song compared to those that did not. Finally, adult exposure to song readily eliminates the effect of song isolation during development. The interesting questions now involve the social, physiological and neurobiological mechanisms regulating the adult plasticity demonstrated by female zebra finches.

CHAPTER THREE

Bailey, D. J., & Wade, J. (2003). Differential expression of the immediate early genes FOS and ZENK following auditory stimulation in the juvenile male and female zebra finch. *Molecular Brain Research, 116*, 147-154.

INTRODUCTION

Regions of the brains of adult zebra finches are tuned to different types of auditory stimuli, although conspecific songs preferentially produce responsiveness. In adult males, neuronal activation indicated by the expression of two immediate early genes, FOS and ZENK, is observed in the caudomedial nidopallium (NCM) and caudomedial mesopallium (CMM; Bolhuis et al., 2001; Jarvis & Nottebohm, 1997; Kruse et al., 2000; Mello et al., 1992; Mello & Clayton, 1994; Mello et al., 1995; Mello & Ribeiro, 1998; Stripling et al., 2001) but not the traditional song control nuclei (Jarvis & Nottebohm, 1997; Mello & Clayton, 1994; Mello & Ribeiro, 1998; Mello et al., 1992) following presentation of their own song or the songs of other conspecifics. Conspecific songs produce more intense effects in the NCM than heterospecific song (Mello et al., 1992; Mello et al., 1995; Kruse et al., 2000), activation that is qualitatively similar in males and females (Mello et al., 1992). I have shown that, in adult female zebra finches, an increased FOS response is seen in not only the NCM, but also the hippocampus following conspecific song but not heterospecific song, tones, or silence (Bailey et al., 2002). FOS expression in the CMM is equivalent in animals presented with conspecific and heterospecific songs, but is significantly increased compared to activation with tone or silence presentations (Bailey et al., 2002).

While much is known about the regions that respond to song in adulthood, less is known about the development of those responses. ZENK expression in juvenile zebra finches has been quantified, but in NCM only (Jin & Clayton, 1997; Stripling et al., 2001). At d20, the ZENK response is constitutively high, but at d30 baseline ZENK is lower and expression is induced significantly by conspecific and heterospecific songs

relative to silence controls (Stripling et al., 2001), although data from males and females were not considered separately. Electrophysiologically, specificity for conspecific song is exhibited by neurons in the NCM of males and females at d30 (Stripling et al., 2001) as in adult males (Chew, Vicario, & Nottebohm, 1996a, 1996b). Therefore, the following experiment examined the expression of the protein products of the immediate early genes FOS and ZENK at 30 days posthatch (d30) to determine whether (1) sex differences in juvenile immediate early gene expression exist; (2) FOS and ZENK are differentially expressed; and (3) the pattern of immediate early gene immunoreactivity in developing male and female zebra finches is similar to that observed in adults. Neuronal activation was assessed at d30 because, in addition to allowing comparisons to my prior work (Bailey et al., 2002; see Chapter 1), the age is critical for song learning in both sexes. That is, it is during the period in which males form templates of their fathers' songs, but before they produce their own (Nordeen & Nordeen, 1997). The experience of female zebra finches at this developmental stage is critical for later responses to song, in that they do not show the normal choice for their father's song over that of other males if isolated from song at d25 (Clayton, 1988), whereas isolation at d35 does not impact their preference (Clayton, 1988; Miller, 1979b).

MATERIALS AND METHODS

Animals and housing

Juvenile male and female zebra finches (d30; day of hatch equals d1; see below for sample sizes) were obtained from the breeding colony at Michigan State University. Animals were housed in communal aviaries consisting of approximately seven breeding

pairs and their young. Free access to seed and water was provided along with once-weekly supplements of spinach or oranges and a mixture of hard-boiled chicken eggs and bread. All stimuli exposures were done during the light portion of the light:dark cycle (12:12; lights on at 7 AM).

Stimulus Exposure and Tissue Collection

Each bird was placed in an individual cage in a darkened, sound isolated room to minimize baseline immediate early gene activity potentially produced by visual stimulation and to replicate conditions of my prior study (Bailey et al., 2002). Following 60 min of acclimation, female-directed male zebra finch song, heterospecific song, randomly generated tones or an empty sound file was played using Cool Edit® (Syntrillium Software Corp.) through a speaker linked to a PC. The sound files and stimulus delivery were the same as in my experiment in which FOS induction was assessed in adult females (Bailey et al., 2002). Zebra finch song was obtained from ten individual males recorded from the breeding colony; heterospecific songs were selected from the National Geographic Society/Cornell Laboratory of Ornithology's Guide to Bird Sounds® and were digitized to play in Cool Edit®. Heterospecific songs were selected based on approximately comparable bout lengths and frequencies overlapping the range of zebra finch song. Species used were American robin, Baird's sparrow, Bell's vireo, Cassin's finch, Connecticut warbler, marsh wren, Scott's oriole, summer tanager, western meadowlark and white breasted nuthatch. Ten tone files approximating the features of zebra finch song were created in Cool Edit®. For each animal, three sound files were randomly chosen from a bank of ten of a particular stimulus condition (except for birds in

the "no song" group, in which one empty sound file was run), based on suggestions for song playback experiments (Kroodsma et al., 2001; Kroodsma, 1989). Each of the three files, 30 sec in length, was repeated in a fixed order, separated by a silence interval of 30 sec for a total stimulus presentation time of 30 min.

One hour following stimulus delivery, while still in the dark, birds were given an overdose of Equithesin. They were perfused with 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Sex was determined post-mortem by examination of the gonads. Brains were removed, post-fixed for 1 hr in 4% paraformaldehyde in PBS, and sunk overnight in 30% sucrose in PBS at 4°C. Three series of frozen, coronal, 30 μm sections were collected from each brain into cryoprotectant, and stored at -20°C until processed for immunohistochemistry.

FOS and ZENK Immunohistochemistry

Tissue was rinsed for one hr in PBS. Then, immunohistochemistry for c-FOS was performed in one set of tissue (as in Bailey et al., 2002) using a chicken primary antibody (1:20,000; D'Hondt, Vermeiren, Peeters, Balthazart, Tlemçani, Ball, Duffy, Vandesande, & Berghman, 1999), a donkey anti-rabbit secondary antibody (1:500; Jackson ImmunoResearch Laboratories) and diaminobenzidine (Sigma) for visualization. ZENK expression was analyzed in a second set of tissue sections from the same animals using the same procedures and reagents, except for the primary antibody (Santa Cruz Biotech; catalog #sc-189; 0.1 μg/ml; Mello & Ribeiro, 1998). Brain sections were mounted on gelatin-coated slides, dehydrated, cleared in xylene and coverslipped with Permount.

Data Analysis

For sections labeled with each antibody, immunoreactive nuclei were counted in the NCM, HP and CMM (as in Bailey et al., 2002). When possible, six sampling areas per brain region for each animal were quantified. However, due to histological artifact, that was not possible for every animal. For analysis of FOS immunoreactivity, an average of 5.3 samples was available for CMM, 5.2 for NCM, and 5.5 for the HP. For ZENK immunoreactivity, the averages were: 4.8 (CMM), 5.3 (NCM), and 4.9 (HP). In a few birds, no sections were available for one of the brain regions, so they were not included in the analysis. Final sample sizes (5-8 per group) are indicated in Figures 3.1 and 3.2. Densities of FOS- and ZENK- immunoreactive nuclei were determined using an ocular grid (as in Bailey et al., 2002) on an Olympus BX60 microscope by a rater blind to treatment condition. Specifically, in the NCM a 0.49 mm high by 0.49 mm wide box was placed at the level of the tractus dorso-arcopallialis (DA), ventrolateral to the HP and dorsal to the dorsal arcopallial lamina (LAD). In the CMM, two sets of counts were taken. Boxes measuring 0.15 mm high by 0.49 mm wide were adjacently placed below the lateral ventricle at the level of the HP, dorsal to the mesopallial lamina (LaM) and medial to the superior frontal lamina (LFS). As in the adult female (Bailey et al., 2002), labeling within these two sampling regions in CMM was nearly identical; the counts were therefore summed and analyzed collectively. Cells were counted in the HP using a 0.196 mm high by 0.49 mm wide box placed neighboring the ventricle and straddling the dorsolateral and dorsomedial subdivisions (Székely, 1999; Székely & Krebs, 1996) of the structure. Densities of FOS- and ZENK-immunoreactive nuclei were calculated for each

bird by taking the average cell counts for each brain region and dividing by its area (square mm).

Effects of sex, auditory stimulus condition and brain region were assessed using analyses of variance (ANOVA) separately for FOS and ZENK (sex and stimulus condition between animals; brain region within). When significant interactions were detected, additional ANOVAs were used to examine effects of stimulus condition within males and females and the effects of auditory stimulus on densities of immunoreactive cells in the individual brain regions. Where appropriate, Fisher's PLSD was used post-hoc for pairwise comparisons. All statistical analyses were performed using Statview™ (SAS Institute).

RESULTS

The most striking results were interactions between sex and stimulus type that were in opposite directions for the two immediate early genes: the induction of FOS and ZENK differed in males and females (Figures 3.1 and 3.2). For FOS (interaction: F (3, 49) = 4.47, p = 0.008), females (F (3, 24) = 3.37, p = 0.035), but not males (F (3, 25) = 2.01, p = 0.138), showed a significant effect of stimulus exposure. For ZENK (F (3, 45) = 2.85, p = 0.049), the effect of stimulus exposure was marginally significant in males (F (3, 22) = 3.05, p = 0.050) but not in females (F (3, 23) = 1.27, p = 0.313; Figure 3.2). Across the NCM, CMM and HP, FOS responses to zebra finch song in females were 200-300% of those detected in the same regions of birds exposed to silence. In contrast, ZENK levels in males in response to zebra finch song were 400-900% of those in the silence-exposed birds. FOS responses in males and ZENK responses in females

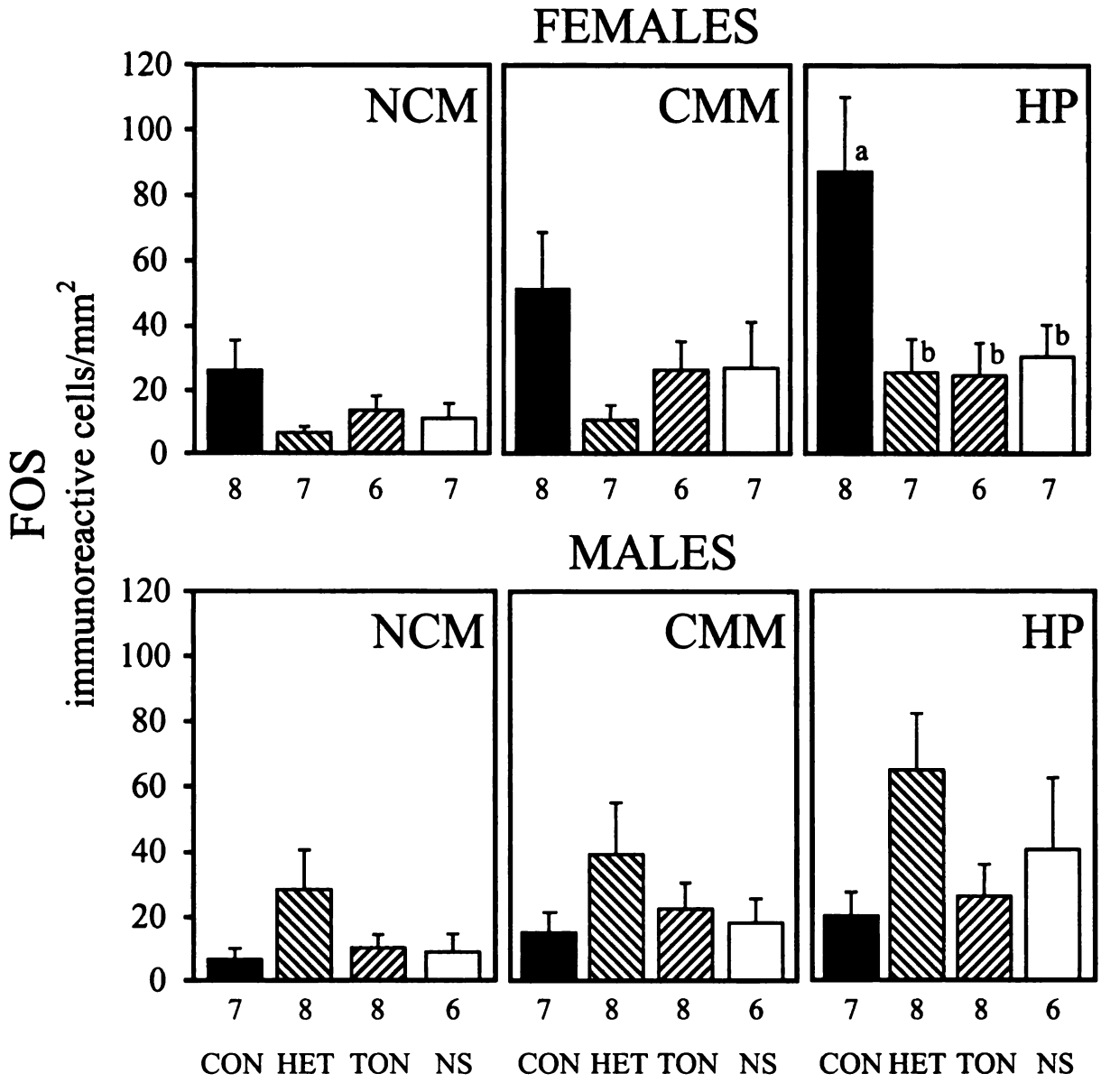


Figure 3.1. Density of FOS-immunoreactive nuclei (mean + SEM) in the caudomedial nidopallium (NCM), caudomedial mesopallium (CMM) and hippocampus (HP) in d30 female and male zebra finches exposed to conspecific (CON) or heterospecific song, tones (TON) or no song (NS). Number of animals analyzed per immediate early gene, brain region and sex is indicated under each bar. Different lowercase letters indicate significant differences between groups within brain region.

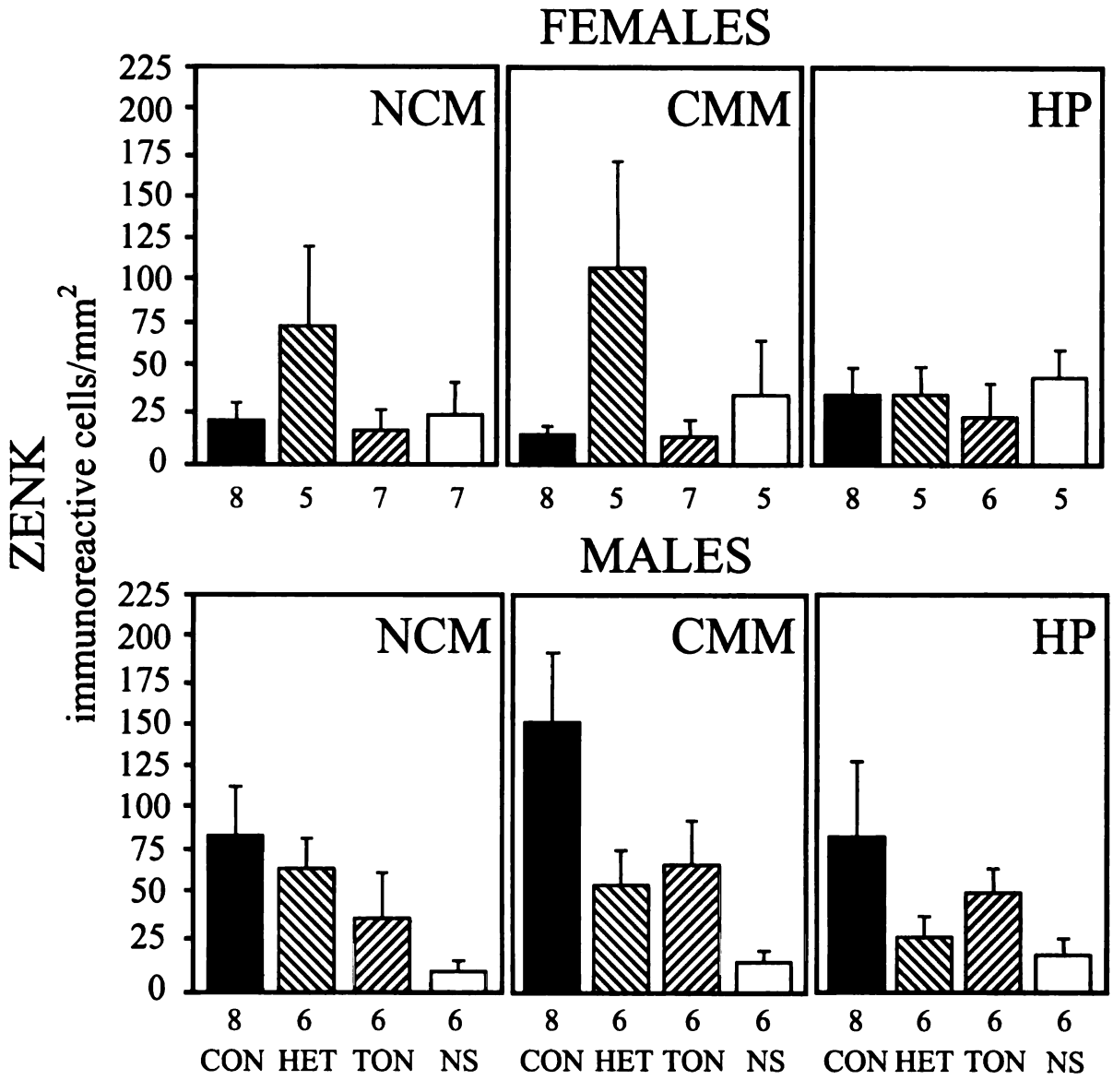


Figure 3.2. Density of ZENK-immunoreactive nuclei (mean +/- SEM) in the caudomedial nidopallium (NCM), caudomedial mesopallium (CMM) and hippocampus (HP) in d30 female and male zebra finches exposed to conspecific (CON) or heterospecific song, tones (TON) or no song (NS). Number of animals analyzed per immediate early gene, brain region and sex is indicated under each bar.

were not increased compared to the matching silence controls (Figures 3.1 and 3.2).

Due to the opposite responses in males and females, main effects of sex and stimulus condition were not detected for either FOS or ZENK (all $F < 1.60$, all $p > 0.206$). However, the brain regions appeared to differ in their responses of both immediate early genes. For FOS (effect of brain region: $F(2, 98) = 19.99$, $p < 0.001$), the density of FOS-immunoreactive nuclei was highest in all areas in females following zebra finch song exposure, but the difference reached statistical significance only the in HP ($F(3, 24) = 4.07$, $p = 0.018$; Fisher's PLSD, $p < 0.014$ for zebra finch song compared to all other stimuli; Figures 3.1 and 3.3). For ZENK, a trend ($F(2, 84) = 3.03$, $p = 0.053$) for the values to be slightly higher in CMM than the other regions was detected in males.

Consistent with the regional differences and the two-way interactions between sex and stimulus type, a three-way interaction of brain region, stimulus condition and sex ($F(6, 84) = 2.34$, $p = 0.039$) was also detected for ZENK immunoreactivity. However, in comparing the two sexes within each of the brain regions, the only significant effect detected was an interaction between sex and stimulus condition in CMM ($F(3, 42) = 4.73$, $p = 0.006$). In males, the highest density of ZENK immunoreactive nuclei was observed following conspecific song, whereas in females, ZENK expression was highest in this region following heterospecific song (Figure 3.2).

Overall, the density of ZENK-immunoreactivity was greater than that observed for FOS (Figures 3.4 and 3.5). As in prior reports in which birds heard song but did not produce it (Bolhuis, Zijlstra, den Boer-Visser, & Van der Zee, 2000; Jarvis & Nottebohm, 1997; Mello & Clayton, 1994; Mello & Ribeiro, 1998; Mello et al., 1992), immediate

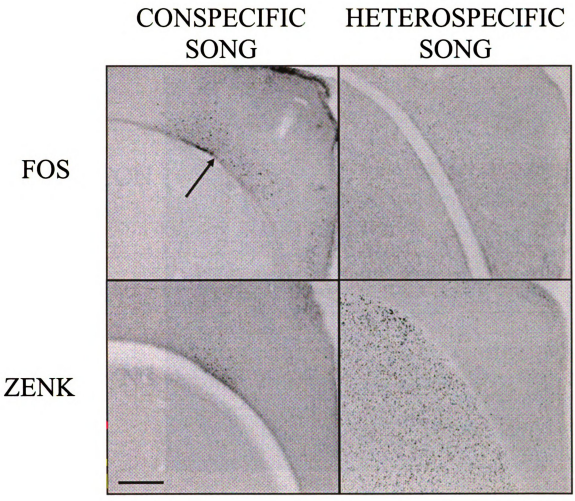


Figure 3.3. FOS- and ZENK-immunoreactivity in the hippocampus (HP) of female zebra finches following conspecific or heterospecific song presentations. The arrow in the top left panel indicates the location of the lateral ventricle, just below where immunoreactive cells are consistently detected. The right edge of each photograph is at the midline. Scale bar = 200 μm.

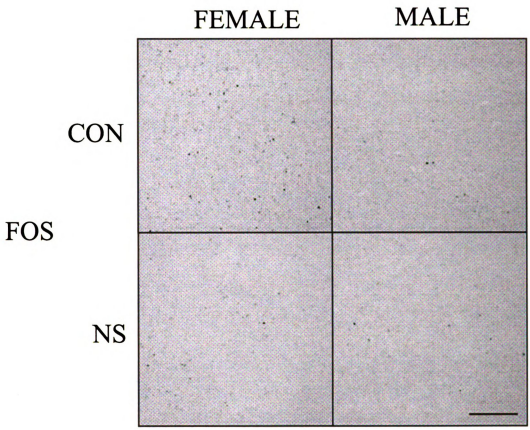


Figure 3.4. Photomicrographs of FOS immunoreactivity in the caudomedial nidopallium (NCM) of female and male zebra finches following presentations of conspecific (CON) or no song (NS). Scale bar = 100 μm.

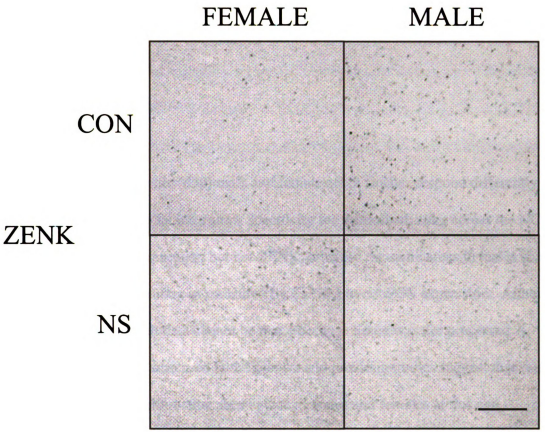


Figure 3.5. Photomicrographs of ZENK immunoreactivity in the caudomedial nidopallium (NCM) of female and male zebra finches following presentations of conspecific (CON) or no song (NS). Scale bar = 100 μm.

early gene activity was not induced in the high vocal center (HVC), robust nucleus of the arcopallium (RA), lateral portion of the magnocellular nucleus of the anterior nidopallium (lMAN) or in Area X (region present in males only).

DISCUSSION

Summary

The brains of juvenile (d30) male and female zebra finches respond differently to auditory stimuli. Juvenile females show specificity for zebra finch song across the NCM, CMM and HP via FOS expression but not ZENK, whereas the same areas in males show specificity for zebra finch song as measured by ZENK but not FOS expression. Although direct comparisons to adult data cannot be made because tissue was not processed concurrently, the patterns observed in the present and previous studies suggest that some aspects of adult perceptual function are in place in males and females at this age. However, it is unclear whether males and females continue to use different patterns of immediate early genes to regulate responses to specific auditory stimuli, which would imply sexually dimorphic neural mechanisms for comparable tasks. In contrast, the responses of the two immediate early genes may equalize in the two sexes as the birds mature.

FOS Expression

The pattern of FOS-immunoreactivity in juvenile females resembles that of adult females. In the present study, presentations of zebra finch song produce a higher density of FOS-immunoreactive neurons across the regions in juvenile females, reaching

statistical significance in the HP. In adult females (Bailey et al., 2002), both the NCM and HP show selectivity for zebra finch song, suggesting that the HP response may develop first. The contribution of the HP to song perception is not yet understood, although FOS activation in response to conspecific song is seen in this region in adults of both sexes (Bailey et al., 2002; Bolhuis et al., 2001; Bolhuis et al., 2000; Kimpo & Doupe, 1997). The response in CMM appears to become less selective as females mature, since in the adult responses to conspecific and heterospecific songs are equivalent (Bailey et al., 2002). In juvenile males, no differences were observed in the densities of FOS-immunoreactive neurons across the auditory stimulus conditions in the areas analyzed. A detailed analysis of FOS expression in the NCM, CMM and the HP under song exposure conditions similar to mine has not been investigated in adult males. So, it is presently unclear whether males lag behind females in acquiring the selective FOS responses or whether this functional difference remains into adulthood.

ZENK Expression

Unlike FOS expression, the density of ZENK-positive cells was increased following exposure to zebra finch song in juvenile males. Qualitatively, this result parallels data from adult male zebra finches: ZENK expression is higher in NCM and CMM following conspecific song presentations than other stimuli (Jarvis & Nottebohm, 1997; Kruse et al., 2000; Mello & Clayton, 1994; Mello et al., 1995; Mello & Ribeiro, 1998; Mello et al., 1992). In contrast, ZENK-immunoreactivity in juvenile females was statistically indistinguishable across the auditory stimulus types in the regions analyzed. These results differ from those of adult zebra finches and other songbirds. In the adult

female zebra finch, ZENK mRNA induction in NCM is qualitatively similar to that seen in males following stimulation with conspecific song; it appears to be higher than that following heterospecific song, tones or silence (Mello et al., 1992). Similarly, presentations of conspecific songs to adult female European starlings produce more ZENK-immunoreactive neurons in NCM relative to silence controls (Duffy et al., 1999).

It is unknown when the response of ZENK-immunoreactivity becomes specific for zebra finch song in the female NCM or whether it does in CMM and the HP. However, like those on FOS expression, the present data on ZENK suggest a sex difference in neural function at d30. One possibility for the conspecific song response in these regions in juvenile males is that ZENK may be important for song template formation during this period. Although song perception is undoubtedly necessary for female zebra finches, and exposure to their fathers' songs at this time seems critical for their preferences in adulthood (Clayton, 1988; Miller, 1979b), they may not form a template in a manner comparable to that of males. The memories necessary for future song production by males may be more specialized than those required solely for recognition, and thus may utilize different neural mechanisms.

ZENK protein in NCM in the present study closely resembles the expression observed by Stripling et al. (Stripling et al., 2001), who report no differences in levels of ZENK mRNA in d30 zebra finches following stimulation with conspecific compared to heterospecific song. The immediate early gene was apparently analyzed for the two sexes combined in their study. When the densities of ZENK-immunoreactive nuclei in the NCM of males and females were considered together, I also detected no difference between the conspecific and heterospecific song groups, and a substantial induction over

the silence control. Thus, although different methods (immunohistochemistry vs. *in situ* hybridization) were used in this study and others (Jin & Clayton, 1997; Stripling et al., 2001), the results for NCM were replicated. Stripling et al. (Stripling et al., 2001) also report using the HP overlying NCM as a control for immediate early gene expression, because it shows a low level of ZENK mRNA. This result, too, appears to be consistent with our data. That is, the ZENK immunoreactivity is localized to a relatively small pocket within the HP which is not directly dorsal to the NCM (Figure 3.3).

General Conclusions

The dorsal telencephalic areas essential to the perception of song, both during development and adulthood, have been determined largely through examination of immediate early gene activity and electrophysiological responses of neurons. The challenge in understanding the function of these brain regions is in pulling these results together. Cells in the NCM of d30 male and female zebra finches show electrophysiological specificity for zebra finch song, and the responses between the sexes is equivalent (Stripling et al., 2001). The same paper, however, describes equivalent ZENK responses to heterospecific and conspecific song, although both are increased compared to silence controls. My immediate early gene data suggest that this conclusion may relate to the pooling of male and female data. Importantly, the present results also suggest that at this juvenile age, the sexes use different patterns of immediate early gene expression to encode information about auditory stimuli. While collectively the electrophysiological and immediate early gene data are consistent with the idea that on average the specific induction of both FOS and ZENK in response to zebra finch song

develops after the specificity of electrophysiological responses, and may be associated with the formation of song memories to some degree in both sexes, it is also possible that the presumably random recordings of cells in NCM (Stripling et al., 2001) were biased toward FOS-expressing cells in females and ZENK-expressing cells in males. Alternatively, the results might reflect the fact that FOS and/or ZENK induction may not always occur in cells that are electrophysiologically active, or that electrophysiological recording is a more sensitive measure than the density of cells expressing immediate early genes. The latter appears to be true in HVC. Significant increases in immediate early gene responses are not detected when a male listens to his own song (Jarvis & Nottebohm, 1997; Mello & Clayton, 1994; Mello & Ribeiro, 1998; Mello et al., 1992), yet discrete peaks of neural activity can be recorded in this area in awake birds during exposure to portions of the song (Nealen & Schmidt, 2002). To help reconcile these ideas, though beyond the scope of this dissertation, it would be worth analyzing immediate early genes in zebra finches exposed to their own song while anesthetized; under these conditions, the electrophysiological responses are robust (Lewicki & Konishi, 1995; Margoliash & Fortune, 1992; Nealen & Schmidt, 2002).

The present data also indicate the value of investigating the responses of both FOS and ZENK at more developmental stages. This song-induced response of ZENK at least is clearly affected by experience. Not only does it develop between d20 and d30, but the onset is delayed in males who are raised without the other members of their clutch (Jin & Clayton, 1997; Stripling et al., 2001). Interestingly, exposure to adult male song is not required (Jin & Clayton, 1997). Questions remain as to whether the FOS response develops in a similar manner, when the sex differences appear, and how long they persist.

While additional experiments are needed to elucidate the details of how neural responses to song develop, this study documents a sex difference in juveniles in the function of cells in brain regions responsive to song. While male and female zebra finches both acquire appropriate responses to song, they appear to do so via divergent mechanisms. This sex difference is intriguing because it exists in areas that are not obviously morphologically distinct. Sexual dimorphisms are very clear in the motor pathway for song production (HVC, RA, nXIIts) and in Area X. For nearly 30 years, birdsong researchers have known that these regions are larger (or only present) in males, who sing, compared to females who do not (Arnold, 1997; Nottebohm & Arnold, 1976; Wade, 2001; Wade & Buhlman, 2000). The differences between the sexes can now be expanded to include areas involved in song perception, at least on a mechanistic level.

CHAPTER FOUR

Bailey, D. J., & Wade, J. (2005). FOS and ZENK responses in 45 day-old zebra finches vary with auditory stimulus and brain region, but not sex. *Behavioural Brain Research, 162*, 108-115.

INTRODUCTION

Communication in zebra finches (*Taeniopygia guttata*) involves the production and perception of a variety of vocal signals by both sexes. The most widely studied of these vocalizations, male song, is learned during a sensitive period in development when, beginning at approximately post-hatch day 25 (d25), sons memorize characteristics of their fathers' songs. Although vocalizations are made by male and female zebra finches shortly after hatching, particularly in the form of begging calls (Zann, 1996), males do not begin to sing their own song until around d45, at which point they actively rehearse it (Johnson, Soderstrom, & Whitney, 2002) to closely match that of their tutor ("sensorimotor integration;" Brainard & Doupe, 2000; Eales, 1985; Immelmann, 1969; Nordeen & Nordeen, 1997). Sensorimotor integration can be delayed or modified by preventing males from making or hearing their own vocalizations (Pytte & Suthers, 2000; Solis, Brainard, Hessler, & Doupe, 2000; Solis & Doupe, 1999). Furthermore, isolation of zebra finch males prior to the sensitive period for song learning until adulthood results in the production of an abnormal song (Eales, 1985; Immelmann, 1969). Females may undergo a similar period for learning as well: acoustic isolation at d25 (Clayton, 1988) eliminates their preference for a father's song over that of another conspecific male, whereas this preference is maintained if isolation begins at d35 (Miller, 1979b).

These results point to the importance, in both sexes, of hearing song during development. Electrophysiological recordings and the measurement of immediate early gene (IEG) responses, like those of c-FOS and ZENK (an acronym for Zif268, Egr-1, NGF1-A, Krox-24), have revealed regions of the brain important for the perception of song. A variety of auditory stimuli elicit activity in brain regions of adult male and

female songbirds outside of the regions that control song learning and production. One of these regions, the caudomedial nidopallium (NCM), responds primarily to conspecific songs (Bailey et al., 2002; Bolhuis et al., 2001; Cheng & Clayton, 2004; Chew et al., 1996a, 1996b; Duffy et al., 1999; Gentner, Hulse, Duffy, & Ball, 2001b; Hernandez & MacDougall-Shackleton, 2004; Jarvis & Mello, 2000; Jarvis & Nottebohm, 1997; Jin & Clayton, 1997; Kruse et al., 2000; Mello & Clayton, 1994; Mello et al., 1995; Mello & Ribeiro, 1998; Mello et al., 1992; Nastiuk, Mello, George, & Clayton, 1994; Park & Clayton, 2002; Phillmore, Bloomfield, & Weisman, 2003; Ribeiro, Cecchi, Magnasco, & Mello, 1998; Stripling, Volman, & Clayton, 1997; Terpstra et al., 2004; Vignal, Attia, Mathevon, & Beauchaud, 2004; Whitney, Soderstrom, & Johnson, 2003). Responses within another auditory perceptual region, the caudomedial mesopallium (CMM), are more variable, but activation similar to that observed in NCM has been shown (Bailey et al., 2002; Bailey & Wade, 2003; Bolhuis et al., 2001; Bolhuis et al., 2000; Cheng & Clayton, 2004; Duffy et al., 1999; Eda-Fujiwara et al., 2003; Gentner et al., 2001b; Hernandez & MacDougall-Shackleton, 2004; Jarvis & Nottebohm, 1997; Kimpo & Doupe, 1997; Kruse et al., 2000; Kruse, Stripling, & Clayton, 2004; Maney, MacDougall-Shackleton, MacDougall-Shackleton, Ball, & Hahn, 2003; Mello & Clayton, 1994; Mello & Ribeiro, 1998; Mello et al., 1992; Park & Clayton, 2002; Phillmore et al., 2003; Sockman, Gentner, & Ball, 2002; Terpstra et al., 2004). FOS and ZENK expression in the zebra finch hippocampus (HP) following conspecific song presentations has also been detected in adult and juvenile males and females (Bailey et al., 2002; Bailey & Wade, 2003; Bolhuis et al., 2001; Bolhuis et al., 2000; Cheng & Clayton, 2004; Eda-Fujiwara et al., 2003; Kimpo & Doupe, 1997; Kruse et al., 2004). However, other

studies examining ZENK have reported little, if any, IEG activity in response to song stimulation in the HP (Jin & Clayton, 1997; Mello & Clayton, 1994), and one published report indicates that electrophysiological responses to auditory stimuli have not been observed in the structure (Chew, Mello, Nottebohm, Jarvis, & Vicario, 1995).

Although a large volume of data from many oscine species details neural responses to auditory stimuli in adulthood, few studies have examined responses to auditory stimuli in the developing zebra finch. In d20 males and females, no ZENK response in NCM is detected following conspecific song presentations relative to a silence control, whereas both conspecific and heterospecific songs (but not tones) induce ZENK expression at d30 (Jin & Clayton, 1997; Stripling et al., 2001). Interestingly, electrophysiological specificity for conspecific song does exist in NCM at d20 and d30 and does not differ between males and females. We have shown that the brains of d30 male and female zebra finches respond differently to auditory stimuli. Overall, across the NCM, CMM and HP, juvenile females show specificity for zebra finch song as measured by levels of FOS- but not ZENK-immunoreactive cells, whereas the same areas in males show specificity for zebra finch song with ZENK but not FOS expression (Bailey & Wade, 2003).

Whether ZENK or FOS continue to be expressed in males and females, respectively, in response to conspecific songs, or whether both IEG responses become equivalent in the two sexes as the birds become adults was unknown. The one study to simultaneously examine IEG activity in adult male and female zebra finches documented only that no qualitative difference existed in ZENK expression in the NCM following conspecific song presentations (Mello et al., 1992); the lack of a difference in the IEG

response mirrors the equivalence in electrophysiological recordings from the NCM of adult males and females in response to novel conspecific songs (Chew et al., 1996b). The specificity of NCM neurons to conspecific songs in adult female zebra finches, measured by FOS-immunoreactivity (Bailey et al., 2002), parallels the distribution of FOS expression in adult males following tutor song exposure (Bolhuis et al., 2001), but novel conspecific songs were not tested in these males. Although direct comparisons of both IEGs in the two sexes are needed, these results suggest that neurons within auditory perceptual regions in adult male and female zebra finches respond to specific auditory stimuli with similar patterns. To further gauge whether song-selective genomic responses in these regions become fine-tuned with developmental experience, the present experiment examined IEG responses at d45 in male and female zebra finches. This point in development was chosen because it is a time when a clear functional difference emerges, in that males but not females are beginning to produce song.

MATERIALS AND METHODS

Animals and housing

Male and female zebra finches at 45 days post-hatching (n = 6 per stimulus condition; see below) were obtained from breeding aviaries at Michigan State University. Birds were provided free access to seed, water, cuttlefish bones and fine gravel along with once-weekly supplements of spinach or oranges and a mixture of hard-boiled chicken eggs and bread. Experiments were initiated during the light portion of the light:dark cycle (12:12; lights on at 0700).

Stimulus Exposure and Tissue Collection

Auditory stimuli and their delivery were the same (except for one minor change indicated below) as used in previous studies from our lab (Bailey et al., 2002; Bailey & Wade, 2003). Each juvenile was placed in an individual cage in a dark, sound isolated room, and following an acclimation period of 60 minutes was presented with either female-directed male conspecific song, heterospecific song or no song. Songs (and in the case of the "no song" group, an empty sound file) were burned onto compact disc and played via a Sony CD Walkman (#D-E220) connected to a speaker located directly in front of the cage (as opposed to PC-delivered song in our prior studies; Bailey et al., 2002; Bailey & Wade, 2003). Conspecific songs were recorded from ten individual males from our breeding colony and were unfamiliar to the birds used in this study. Heterospecific songs (American robin, Baird's sparrow, Bell's vireo, Cassin's finch, Connecticut warbler, marsh wren, Scott's oriole, summer tanager, western meadowlark and white breasted nuthatch) were selected from the National Geographic Society/Cornell Laboratory of Ornithology's Guide to Bird Sounds. Each bird was presented three conspecific or heterospecific song files randomly chosen from a bank of ten of each stimulus type, except for those in the "no song" group to which one 30 minute empty sound file was played. The conspecific and heterospecific song groups received 30 minutes of auditory stimulation with each of the three respective song files, all 30 seconds in length, repeated in a fixed order and separated by silence intervals of 30 seconds. Birds were euthanized one hour following the end of stimulus delivery (see below).

In order to determine whether any movements or vocalizations made by the birds could have confounded the interpretation of our results, a one hour tape recording was made from each bird that captured the 30 minutes of stimulus exposure and the following 30 minutes. No male sang during this period, although one male and two females presented with conspecific songs called at the initiation of the stimuli. The male called four times and the two females one and six times, respectively. No calls were made by any other bird. In addition, no movements that were audible to the experimenter were made by any of the birds.

Birds were injected with an overdose of Equithesin and perfused with 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Although at this age the plumage of male and female zebra finches has begun to differentiate, the gonads were examined post-mortem to confirm the sex of each bird. Brains were removed, post-fixed for 1 hr in 4% paraformaldehyde in PBS, sunk overnight in 30% sucrose in PBS at 4°C, cut frozen into three series of 30 μm coronal sections, and stored at -20°C in cryoprotectant until immunohistochemistry.

FOS and ZENK Immunohistochemistry

Immunohistochemical procedures were the same as those used previously (Bailey et al., 2002; Bailey & Wade, 2003). Briefly, following the rinsing of cryoprotected tissue with PBS, the protein products of c-FOS and ZENK were visualized using separate, alternate sets of tissue. For FOS, a chicken primary antibody (1:20,000; D'Hondt et al., 1999), a donkey anti-rabbit secondary antibody (1:500; Jackson ImmunoResearch Laboratories) and diaminobenzidine (Sigma) were used. For ZENK, the same procedures

and reagents were used, except for the primary antibody (Santa Cruz Biotech; catalog #sc-189; 0.1 μg/ml). Following protein visualization, brain sections were mounted on gelatin-coated slides, dehydrated, cleared in xylene and coverslipped with Permount.

Data Analysis

FOS and ZENK immunoreactivity were determined in the NCM, CMM and HP of each bird from six sampling areas per brain region, when available. For FOS, the averages were 5.7 sections for NCM and 5.2 each for the CMM and HP; for ZENK, the averages were: 5.7 (NCM), 4.9 (CMM) and 5.1 (HP). FOS- and ZENK-immunoreactive nuclei were counted within an ocular grid on an Olympus BX60 microscope by an observer not aware of auditory stimulus conditions or sex of the animals. Cells within NCM were counted two ways. First, to replicate analyses of immunoreactivity observed in prior studies (Bailey et al., 2002; Bailey & Wade, 2003), a 0.49 mm high by 0.49 mm wide box was placed approximately 0.2 mm from the midline at the level of the dorsal arcopallial tract (DA), ventrolateral to the HP and dorsal to the dorsal arcopallial lamina (LAD). Recently, however, the analysis of ZENK immunoreactivity in medial and lateral divisions of NCM has been separately evaluated in response to a bird's own song, tutor song, or that from a novel conspecific (Terpstra et al., 2004). Therefore, cells within these regions were then counted in two individual boxes 0.49 mm high by 0.49 mm wide placed at the same dorsal-ventral level of the brain as described above. For cell counts within medial NCM, one edge of the grid was placed at the midline of the telencephalon; for those in the lateral NCM, the medial edge was placed next to the first, 0.5 mm from the midline. As in our previous studies (Bailey et al., 2002; Bailey & Wade, 2003), cells

within the CMM were counted in two boxes measuring 0.15 mm high by 0.49 mm wide adjacently placed below the lateral ventricle at the level of the HP, dorsal to the mesopallial lamina (LaM) and medial to the superior frontal lamina (SFL). Similar to data obtained from adult females (Bailey et al., 2002) and d30 juvenile males and females (Bailey & Wade, 2003), labeling within these two sampling regions in CMM was nearly identical, so the counts were summed and analyzed collectively. In the HP, cells were counted using a 0.196 mm high by 0.49 mm wide box placed neighboring the ventricle across regions defined as "dorsolateral" and "dorsomedial" (Székely, 1999; Székely & Krebs, 1996).

Average cell counts for each brain region were divided by ocular grid area (in square mm; NCM = 0.240, CMM = 0.0735 + 0.0735 = 0.147 and HP = 0.096) to determine densities of FOS- and ZENK-immunoreactive nuclei. Analyses of variance (ANOVAs) were used to determine the effects of sex (between individuals), auditory stimulus condition (between individuals), and brain region (within individuals) independently for FOS and ZENK, using measurements from NCM (the first analysis described above), CMM and the HP. A separate ANOVA was used to determine differences between males and females and auditory stimulus conditions in the medial and lateral NCM (within individuals). For each analysis, Fisher's PLSD was used post-hoc as appropriate for pairwise comparisons when significant main effects were detected. Statistical analyses were performed using Statview (SAS Institute).

RESULTS

Significant main effects of auditory stimulus condition (ZENK: F (2, 30) = 3.64, p = 0.038; FOS: F (2, 30) = 4.87, p = 0.015) and brain region (ZENK: F (2, 60) = 19.79, p < 0.001; FOS: F (2, 60) = 6.36, p = 0.003) were found for both IEGs analyzed. No main effect of sex for either IEG was detected (ZENK: F (1, 30) = 0.39, p = 0.535; FOS: F (1, 30) = 1.90, p = 0.179). No interactions between sex and auditory stimulus condition were found for either ZENK (F (2, 30) = 0.28, p = 0.755) or FOS (F (2, 30) = 0.81, p = 0.453), and there were no significant two- or three-way interactions among the variables measured (all F < 1.51, p > 0.209). Across the brain regions, ZENK and FOS immunoreactivity were increased following conspecific song presentations (both sexes combined; ZENK: F (2, 33) = 3.88, p = 0.031; FOS: F (2, 33) = 4.79, p = 0.015; Figures 4.1 and 4.2). However, the details were slightly different for the two IEGs. For ZENK, immunoreactivity following zebra finch song presentations differed only from silence (Fisher's PLSD p = 0.009), whereas for FOS, conspecific song increased IEG expression compared to both other stimuli (p < 0.016). Similarly, while the HP consistently showed the lowest response (ANOVA, main effect of brain region with combined sexes; ZENK: F (2, 66) = 20.07, p < 0.001; FOS: F (2, 66) = 6.36, p = 0.003), it differed significantly from both the NCM and CMM in the ZENK analysis (p < 0.001) but only from the CMM in the FOS analysis (p = 0.001). Interestingly, FOS expression in the CMM only was higher than in the NCM (p = 0.008), an effect likely due to increased basal expression rather than an enhanced specific response (Figure 4.1). The levels of ZENK and FOS expression in these regions in the two females and one male that called during

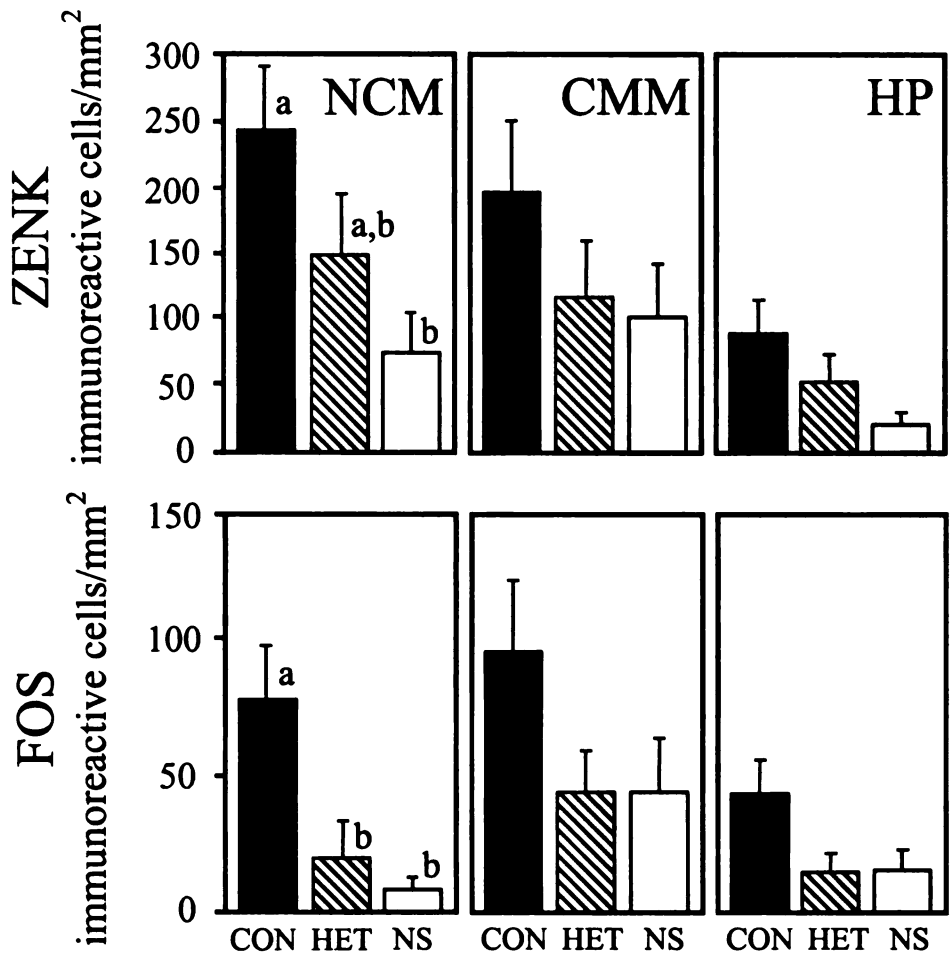


Figure 4.1. Density of ZENK- (top panels) and FOS- (bottom panels) immunoreactive neurons in the caudomedial nidopallium (NCM), caudomedial mesopallium (CMM) and hippocampus (HP) of 45 day post-hatch male and female zebra finches following presentations of conspecific songs (CON), heterospecific songs (HET) or no song (NS; n = 6 males and n = 6 females for each). Different lowercase letters indicate significant differences between groups within brain region and IEG. Note the y-axis scale for ZENK immunoreactivity is greater than that for FOS.

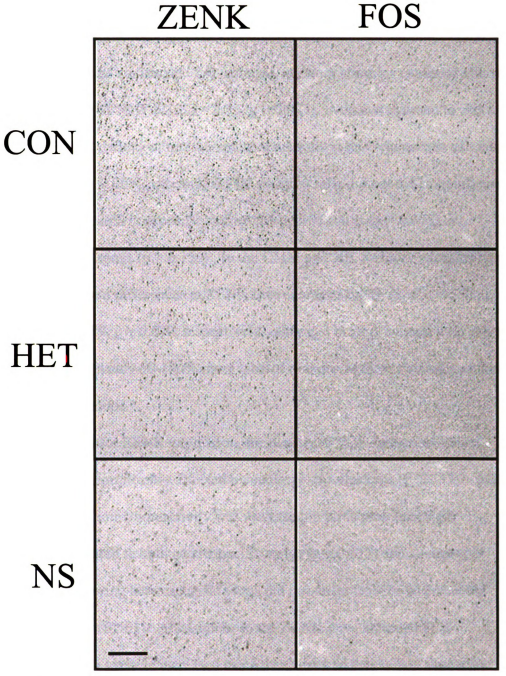


Figure 4.2. Photomicrographs of ZENK- (left panels) and FOS- (right panels) immunoreactivity in the caudomedial nidopallium (NCM) of male or female zebra finches following presentations of conspecific songs (CON), heterospecific songs (HET) or no song (NS). Scale bar = 100 μm.

conspecific song presentations did not noticeably differ from the means of their respective groups.

The response patterns were generally similar among the brain regions, but the magnitude of effects varied somewhat. For example, auditory stimulus exposure affected ZENK levels within the NCM (F (2, 33) = 4.05, p = 0.027). Animals exposed to zebra finch songs had a higher density of immunoreactive neurons in this region than animals not exposed to song (p = 0.008), although ZENK-positive cells did not differ significantly between birds presented with conspecific and heterospecific songs (p = 0.173) or heterospecific versus no songs (p = 0.156). In the CMM and HP, auditory stimulus type did not produce significant differences in ZENK immunoreactivity (F (2, 33) = 2.33, p = 0.113 and F (2, 33) = 2.89, p = 0.070, respectively), although birds presented with zebra finch songs had approximately 50-100% more labeled neurons in these two regions than those in the other two groups.

Similar to results for ZENK expression, the density of FOS-immunoreactive nuclei in the NCM was significantly affected by auditory stimulus type (F (2, 33) = 6.26, p = 0.005). In this case, immunoreactivity was increased in birds presented with conspecific songs compared to both heterospecific songs (p = 0.012) and silence (p = 0.002). Responses following heterospecific songs did not differ from birds presented with no song stimuli (p = 0.501). Also similar to the ZENK data, although FOS immunoreactivity was on average increased more than two-fold following conspecific songs, auditory stimulus condition did not result in significant differences in expression in the CMM (F (2, 33) = 2.02, p = 0.149) or HP (F (2, 33) = 2.31, p = 0.115).

In both the medial and lateral NCM, conspecific song presentations produced the highest IEG responses (Figure 4.3); a significant effect of auditory stimulus condition was found for FOS (F (2, 30) = 4.55, p = 0.019) and approached significance for ZENK (F (2, 30) = 3.10, p = 0.060). Overall, both ZENK (F (1, 30) = 21.60, p < 0.001) and FOS (F (1, 30) = 13.52, p = 0.001) immunoreactivity differed significantly between lateral and medial NCM, with levels higher in the lateral than medial portion for both IEGs. Densities of ZENK- (F (1, 30) = 0.08, p = 0.785) or FOS- (F (1, 30) = 0.97, p = 0.332) immunoreactive nuclei did not significantly differ between males and females, nor were interactions between sex and auditory stimulus condition uncovered (ZENK: F (2, 30) = 0.59, p = 0.559; FOS: F (2, 30) = 0.74, p = 0.486). None of the two- or three-way interactions were significant (all F < 2.67, p > 0.085).

Based on the lack of a sex difference and interactions, data from males and females were pooled to further examine differences between the lateral and medial portions of NCM, and the results were statistically identical to those above. For both IEGs, expression was more dense in the lateral than medial NCM (Figure 4.3; ZENK: F (1, 33) = 22.08, p < 0.001; FOS: F (1, 33) = 13.73, p = 0.001). The effect of stimulus condition approached significance for ZENK (F (2, 33) = 3.27, p = 0.051) and differed for FOS expression (F (2, 33) = 4.63, p = 0.017). No significant interaction between stimulus type and NCM region was found for either ZENK (F (2, 33) = 2.73, p = 0.080) or FOS (F (2, 33) = 1.36, p = 0.270). Conspecific song presentations resulted in significantly more ZENK-positive cells in the combined lateral and medial NCM than birds not exposed to song (Fisher's PLSD p = 0.015), but not significantly more than those resulting from heterospecific songs (p = 0.251). ZENK-immunoreactivity

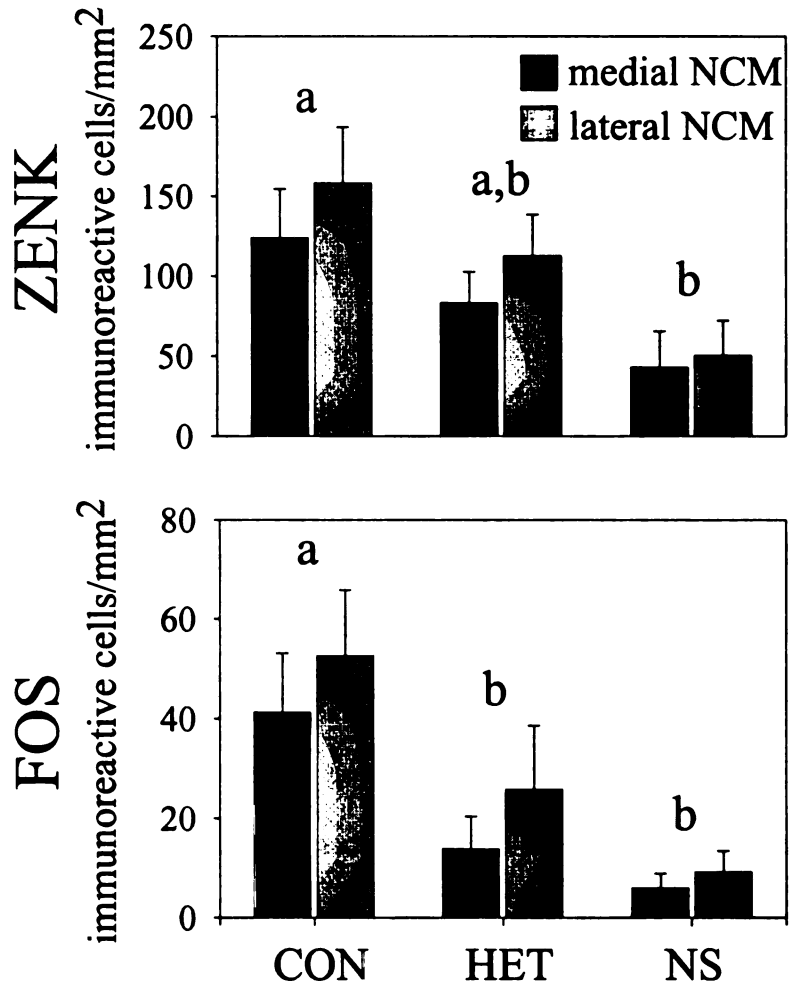


Figure 4.3. ZENK- (top graphs) and FOS- (bottom graphs) immunoreactive nuclei in the medial and lateral caudomedial nidopallium (NCM) following conspecific (CON), heterospecific (HET) or no song (NS) presentations. For both ZENK and FOS, expression within lateral NCM was significantly greater than that in medial NCM (see Results). Different lowercase letters in each graph indicate significant differences between auditory stimulus conditions within these regions. Note the y-axis scale for ZENK immunoreactivity is greater than that for FOS.

following heterospecific songs did not differ from that observed in silence controls (p = 0.175). For FOS, conspecific songs produced more immunoreactive nuclei than heterospecific songs (p = 0.049) and no song (p = 0.006); immunoreactivity between groups exposed to heterospecific songs and no song did not significantly differ (p = 0.360).

DISCUSSION

The expression of ZENK- and FOS-immunoreactive neurons to auditory stimuli was sexually monomorphic in d45 zebra finches, but varied across the brain regions analyzed and the type of auditory stimulus presented. Conspecific song produced the highest densities of ZENK and FOS immunoreactivity across the NCM, CMM and the HP. Although the pattern of expression was the same for all regions analyzed, overall ZENK expression in the HP was lower than that in the NCM and CMM. Significant differences in IEG expression produced by auditory stimuli were found only in the NCM, and within the structure the expression of ZENK and FOS was higher in the lateral compared to the medial portion.

The similar response in the two sexes is probably more like adults (see introduction to this chapter) and differs qualitatively from the pattern we detected at d30. At that age, across the NCM, CMM and HP, males express increased ZENK but not FOS, and females show the reverse, in response to conspecific songs. Although direct comparisons to this prior work cannot be made because tissue was not processed concurrently, at d45 the FOS and ZENK responses in both sexes are higher following conspecific songs than the other auditory stimulus conditions; the sex difference observed

in animals 15 days younger has disappeared. Regarding the selectivity of responses, others have found no effect of stimulus type in the ZENK responses of NCM cells at d20, but conspecific and heterospecific song increased ZENK expression relative to basal levels at d30 (Stripling et al., 2001). In our study using d30 birds (Bailey & Wade, 2003, Chapter 3) conspecific songs produced the highest densities of IEGs in all regions analyzed, but the effect was only statistically significant in the HP using the FOS analysis, and marginally significant in the CMM for ZENK. In the present study, significant effects of auditory stimulus condition are found only within the NCM for both IEGs, which is the one effect consistently detected over all studies of this type on adults (see Introduction to this chapter). Also, in contrast to what was seen in d30 birds (Bailey & Wade, 2003), the density of ZENK- expressing cells in NCM is higher than that in the HP, and FOS-immunoreactivity in the two regions is equivalent, similar to what is seen in adult females (Bailey et al., 2002). Therefore, it appears that birdsong begins to selectively induce IEGs between d20-d30 but, by d45, FOS and ZENK responses begin to be sharpened. These results parallel those from another experiment at d45, in which the NCM in both males and females exposed to normal conspecific song showed two times more ZENK-immunoreactive neurons than birds presented with abnormal song (Tomaszycki, Sluzas, Newman, Adkins-Regan, & DeVoogd, 2004). Thus, as in adults, neurons within NCM at this age respond based on song characteristics and quality.

FOS- and ZENK-immunoreactivity in response to novel song playback (and other auditory stimulus conditions) in the present study was greatest in the lateral but not medial portion of NCM, contrary to data recently reported in Terpstra et al. (Terpstra et al., 2004) for adult songbirds. Interestingly, despite the increased responses in the medial

NCM, a significant positive correlation between ZENK immunoreactivity and the number of song elements copied from a tutor was found in the lateral but not medial NCM. This effect was detected when tutor song was presented; no significant correlation between number of elements copied and immunoreactivity following a bird's own song or a novel song was uncovered (Terpstra et al., 2004). Furthermore, a marginally significant negative correlation between ZENK immunoreactivity in medial NCM and number of copied elements was also found (Terpstra et al., 2004). In conjunction with the present data, these findings may suggest that the lateral region of NCM plays a role in acquiring or utilizing the song template. That is, at d45 the lateral NCM may show an increased IEG response to conspecific song because of enhanced activity associated with comparing zebra finch vocalizations to the template during sensorimotor integration. In contrast, this function is diminished in adults.

The functional significance of song-induced IEG activation in the HP and CMM also requires further investigation. A number of studies from several labs have detected variable responses in the HP in terms of quantity of IEG labeling, whether FOS or ZENK is measured, whether the response is specific to particular auditory stimuli, and perhaps the number of song stimuli used. In adult female zebra finches, conspecific songs produce a higher density of FOS-immunoreactive neurons in the HP than heterospecific songs, tones or no songs (Bailey et al., 2002). In d30 females, FOS-positive cells are highest in the HP following zebra finch song exposures compared to heterospecific songs, tones or silence (Bailey & Wade, 2003). Other studies have reported IEG activation in the HP in response to auditory stimulation with novel conspecific songs and tutor song (Bolhuis et al., 2001; Bolhuis et al., 2000; Eda-Fujiwara et al., 2003; Kimpo & Doupe,

1997) and by the pairing of conspecific song with multi-colored lights (Kruse et al., 2004). Compared to silence controls, male zebra finches presented with conspecific song stimuli also showed a 40% increase in phosphorylated extracellular-signal regulated kinase (pERK) in the HP, which is integral to ZENK gene activation (Cheng & Clayton, 2004). Other studies have stated explicitly that no IEGs are observed in the HP following song presentation (Mello & Clayton, 1994; Stripling et al., 2001), and another normalized staining in the NCM to the HP (Jin & Clayton, 1997), suggesting minimal IEG activity within the structure. Although it has been reported that electrophysiological responses to auditory stimuli are not found in HP neurons (Chew et al., 1995), auditory responses have been observed in some HP cells using conspecific song and white noise as stimuli to examine spike-rate discrimination in the zebra finch (Dr. Mark Hauber, personal communication). It is clear that the HP in some cases responds to conspecific song but studies must be designed to evaluate the specific nature of that response, including the extent of its anatomical distribution (see Bailey et al., 2002; Bailey & Wade, 2003).

IEG responses in the CMM are also highly variable. For example, cells in the CMM are active in response to a bird's own song (Jarvis & Nottebohm, 1997; Kimpo & Doupe, 1997), tutor song (Bolhuis et al., 2000), novel conspecific song (when it is the only stimulus tested; Duffy et al., 1999; Kimpo & Doupe, 1997; Kruse et al., 2000, 2004; Mello & Ribeiro, 1998), song of a familiar rather than foreign dialect (Maney et al., 2003) and the length of song stimuli (Sockman et al., 2002). Some studies have shown that the CMM may, in particular species and at certain points in development, respond more to conspecific than heterospecific songs (Bailey & Wade, 2003; Hernandez & MacDougall-Shackleton, 2004), and others have observed more general auditory responses (Bailey et

al., 2002; Bailey & Wade, 2003; Eda-Fujiwara et al., 2003; Gentner et al., 2001b; Mello et al., 1992; Phillmore et al., 2003; Terpstra et al., 2004). Similarly, the density of IEG expression in response to auditory stimuli has been reported as both greater (Bailey et al., 2002; Bailey & Wade, 2003; Bolhuis et al., 2001; Bolhuis et al., 2000; Gentner et al., 2001b; Hernandez & MacDougall-Shackleton, 2004; Maney et al., 2003) and less (Phillmore et al., 2003) in the CMM than NCM. It is clear that, as for the HP, additional work is needed to clarify the role of the CMM. Still, the results of the present report document a maturation of the perceptual regions, NCM in particular, such that by d45 several adult-like characteristics are in place in both males and females.

CHAPTER FIVE

Afferent connectivity of the zebra finch hippocampus determined by iontophoretic injections of the retrograde tracer fast blue

INTRODUCTION

Zebra finches learn, produce and respond to vocal signals via regions that are linked in generally well-defined neuronal circuits. The regions important for song template acquisition and the interconnections with the motor control circuitry integral for sensorimotor integration and song production in male zebra finch vocalization are well delineated (see Chapter 1; Bottjer et al., 2000; Mello et al., 1998; Vates et al., 1996; Vates & Nottebohm, 1995; Vates et al., 1997). In addition, connections among other telencephalic structures, specifically the caudomedial nidopallium (NCM), caudomedial mesopallium (CMM) and Field L (homologous to mammalian primary auditory area; receives information from auditory thalamus, at least in males; Vates et al., 1996), have been described and are consistent with the idea that the regions work together to process song information.

Expression of the protein products of the immediate early genes c-FOS and ZENK (to some extent) in the hippocampus following conspecific songs only (Chapters 3 and 4; Bailey et al., 2002; Bailey & Wade, 2003) suggests that the region may be involved in auditory discrimination or memory formation related to song behavior. However, direct connections from auditory perceptual areas to the hippocampus have not been documented in the zebra finch brain. Connections exist that could allow the hippocampus to be involved in the processing of auditory information, specifically to the dorsomedial portion of the thalamic nucleus (Székely & Krebs, 1996; DMP), part of a "thalamo-cortical" circuit potentially involved in song learning or perception (Vates et al.,

1997), as well as portions of the arcopallium, perhaps including neurons associated with the motor nucleus RA or its "cup" (Székely & Krebs, 1996). However, afferents to the hippocampus must be determined to fully understand how the region may interact with other areas known to play a role in song perception, production, or memory formation. Together with data already published detailing the efferent connectivity of the structure (Székely & Krebs, 1996), the afferent connections of the hippocampus were investigated here to enable the generation of a working model of hippocampal circuitry in male and female zebra finches to allow us to better understand its role in song-related behavior, and to examine potential structure and function relationships with homologous pathways in mammals. Injections were made only into the dorsolateral subdivision of the hippocampus (DLHP) to mirror the site of injection of an anterograde tracer in a prior study (Székely & Krebs, 1996) and because the DLHP is the region of the hippocampus where immediate early gene activity is most robust following conspecific song presentations (see Figure 3.3).

MATERIALS AND METHODS

Animals and housing, tracer injection and tissue preparation

Adult male (n = 4) and female (n = 4) zebra finches were anesthetized with isoflurane. Glass micropipettes were prepared from borosilicate glass capillaries (World Precision Instruments, Inc., Sarasota, FL) using a Vertical Micropipette Puller (Sutter Instrument Co., Novato, CA). The tip of each micropipette was broken to a diameter of 10-12 μm, and fitted in the electrode holder of a stereotaxic frame (David Kopf Instruments, Tujunga, CA). Birds were mounted in the stereotaxic apparatus with head

level in a beak clamp (two points picked along sagittal suture and their ventral coordinates measured and equalized; bar adjusted as necessary). The skull was exposed, and the intersection of the lambdoidal and sagittal sutures was measured. A small pencil mark was then made at coordinates relative to that intersection point for injections into the hippocampus (anterior/posterior (AP) = +1.0, lateral (L) = +/- 0.5) and/or CMM (for confirmation of afferent HP connectivity (see Results); AP = +1.8, L = +/- 1.5).

Using a 1 mm diameter engraving cutter in a Dremel® rotary tool (S-B Power Tool Co., Racine, WI), a hole was made at the coordinates indicated above. The dura mater underneath this hole was carefully teased apart, and if necessary blood was removed with cotton swabs or GelFoam® (Pharmacia and Upjohn Co., Kalamazoo, MI). A small volume of fast blue (0.5 µl; 30 µg/ml saline; Illing Plastics, Bergfeld, Germany) or fluorogold (0.5 µl; 3% + 1% dimethylsulfoxide (DMSO) in 0.1 M phosphate buffered saline (PBS); Fluorochrome, Inc., Englewood, CO) was transferred to each glass micropipette by capillary action or suction. Excess tracer on the outside of the micropipette was absorbed with a cotton swab prior to injection. A silver wire electrode was lowered into the micropipette, and the positive output lead of a Midgard™ Precision Current Source (Stoelting Co., Wood Dale, IL) was attached to it. A 'ground' output lead from the current source was attached to a skin flap overlying the skull. The current source was turned on, and the output current adjusted to 7 µA. Micropipettes were lowered through the hole in the skull to a ventral (V) depth of -0.3 relative to the surface of the brain for the hippocampus and -0.5 for the CMM. Alternating (7 seconds on, 7 seconds off) current was used to unilaterally iontophorese the retrograde tracer(s) into the

region(s). After 10 min, the alternating output switch was turned off, and the micropipette remained in place for 10 min before removal.

The hole in the skull was covered with bone wax (Fine Science Tools Inc., Foster City, CA) and the flaps of skin reconnected with silk suture (Ethicon, Inc., Somerville, NJ) and sealed with collodion (Mallinckrodt Baker, Inc., Phillipsburg, NJ). Birds were removed from the stereotaxic apparatus and then placed individually in cages in the main bird colony with free access to food, water and supplements of spinach or oranges and hard boiled chicken eggs and bread.

Five days following tracer injection (survival time based on pilot work), each bird was overdosed with Equithesin and perfused transcardially with 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Brains were post-fixed for 1 hr in 4% paraformaldehyde and then sunk overnight in 30% sucrose in PBS while continuously protected from exposure to light.

Brains were cut under dim light coronally on a cryostat at 30 μm and mounted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Two alternate sets were cut. One set from each zebra finch was rapidly dehydrated, cleared in xylene, and coverslipped with DPX (Sigma-Aldrich, St. Louis, MO). Tissue remained in the dark while drying. The alternate set of tissue from each bird was stained with thionin, dehydrated, cleared with xylene, and coverslipped with Permount to aid identification of the locations of administration of the tracers and labeled cells, as well as any potential damage to the tissue due to the injection.

Data Analysis

Under fluorescent light, the injection site and cells labeled with fast blue and fluorogold were digitally photographed using PictureFrame (version 2.2) software and a MacroFIRE digital camera (model #S99831; Optronics, Goleta, CA) connected to an Olympus BX51 microscope. Images in this chapter of the dissertation are presented in color. Labeling in a brain region was determined to be absent (no filled neurons), low (1-5 labeled cells per tissue section), moderate (6-10 labeled cells) or high (> 10 cells).

RESULTS

Birds with injection sites limited to within or just above the area of the DLHP in which FOS-immunoreactive cells were observed following conspecific song presentations (Bailey, Rosebush & Wade, 2002; Bailey & Wade, 2003) were included in the analysis. Retrogradely labeled cells from injections medial or lateral to this region or that penetrated other portions of the telencephalon ventral to the lateral ventricle were not examined in detail. Injection sites were typically 100-200 μm in diameter (e.g., Figure 5.1) and were determined by the largest diameter of fast blue product (designated by a brown color under the wavelength of fluorescent light used) in a series of coronal sections. Labeled cells were clearly visible clustered around the injection sites, and the iontophoretic injections exhibited no observable leakage around the micropipette path.

A summary of the main brain regions in which retrogradely labeled cells were observed is presented in Table 1. In both male and female zebra finches, retrogradely labeled neurons were consistently detected in the dorsomedial (DMHP) and ventral (VHP) subdivisions of the hippocampus, CMM (Figure 5.1) and lateral septum (SL; Figure 5.2).

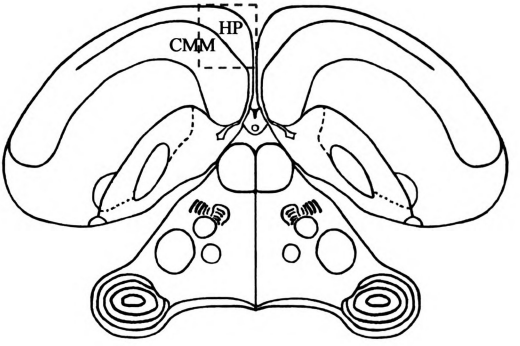
In some cases, the projections seemed to be sexually dimorphic. In females but not males, cells filled with fast blue were found in the periventricular nucleus of the hypothalamus (PVN; Figure 5.2), and in males but not females, labeled cells were detected in the posterior portion of the dorsomedial thalamic nucleus (DMP; Figure 5.3). In all but one of the males and in only one female, retrogradely labeled cells were found in HVC (Figure 5.4). In addition to these major projections, a few cells were observed (inconsistently among the animals) in nucleus taeniae (Tn), the rostromedial portion of the medial striatum (MSt), nucleus rotundus (Rt), the pedunculopontine tegmental nucleus (TPc), and the Substance P reactive nucleus (SPf; data not shown).

To begin to confirm the afferent connectivity of CMM with the hippocampus, fast blue was injected into the hippocampus and fluorogold into the CMM of a male zebra finch (Figures 5.5A and B, respectively). In the brain of this animal, cells filled with fast blue were detected in the CMM (Figure 5.5C), and fluorogold-labeled neurons were observed in the hippocampus (Figure 5.5C) throughout the subdivisions (Figure 5.5D). Results similar to these were obtained when fast blue was iontophoresed into the CMM of a female zebra finch: retrogradely labeled cells were observed in the DLHP and DMHP (data not shown).

	Number of birds and relative density of fast blue labeled neurons	
Region	Males	Females
DMHP	4/4 ++	3/3 ++
VHP	4/4 ++	2/3 ++
CMM	3/4 +++	3/3 +++
SL	4/4 +	2/3 +
PVN	0/4 -	3/3 ++
DMP	3/4 +	0/3 -
HVC	3/4 ++	1/3 ++

Table 5.1. Brain regions in male and female zebra finches exhibiting fast blue labeled neurons following iontophoresis into the dorsolateral subdivision of the hippocampus (DLHP). Below the number of birds of each sex in which retrogradely labeled neurons were observed (or not) in a particular brain region, the density of labeling is indicated as follows: +++ (high number of labeled neurons); ++ (moderate number of labeled neurons); + (few labeled neurons); - (no labeled neurons). DMHP: dorsomedial subdivision of the hippocampus; VHP: ventral subdivision of the hippocampus; CMM: caudomedial mesopallium; SL: lateral septum; PVN: periventricular nucleus; DMP: posterior portion of the dorsomedial nucleus of the thalamus.

Figure 5.1. Coronal section through the dorsomedial surface of the zebra finch telencephalon (top; red box indicates photographed area in bottom panel) at the level of the hippocampus showing iontophoresed fast blue in the dorsolateral (DLHP) subdivision. Note the retrogradely labeled cells at each arrow in the dorsomedial (DMHP) and ventral (VHP) subdivisions of the structure and in the caudomedial mesopallium (CMM). Scale bar = 200 μm.


HP
CMM

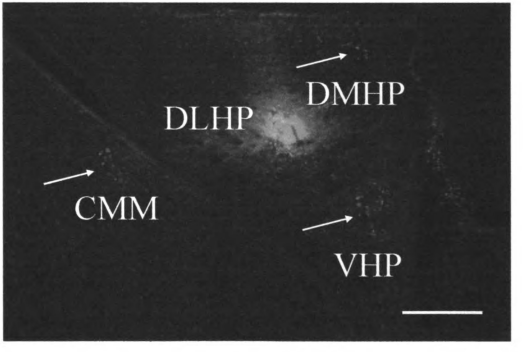
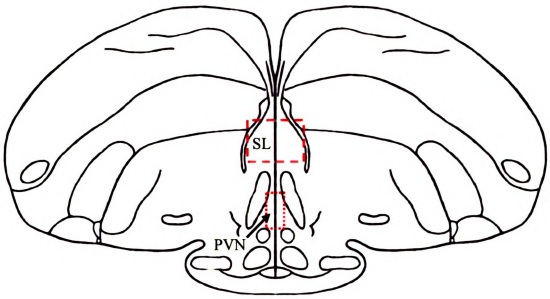
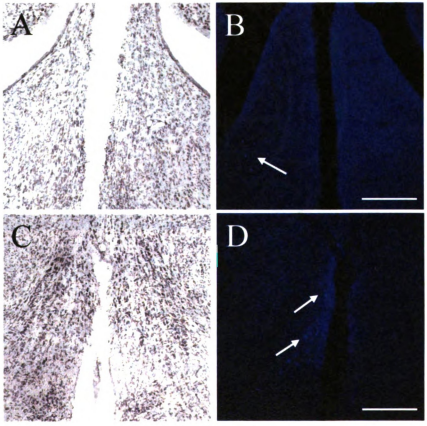

DMHP
DLHP
CMM
VHP

Figure 5.2. Coronal sections through the zebra finch brain at the level of the lateral septum (SL; dashed red box) and periventricular nucleus of the hypothalamus (PVN; dotted red box). Iontophoretic injections of fast blue into the dorsolateral subdivision of the hippocampus resulted in ipsilaterally labeled cells within the SL (arrows, panel B; photo from a male zebra finch) in both male and female zebra finches and the PVN in females only (arrows, panel D). Thionin stained tissue through the SL (panel A) and PVN (panel C) are provided for comparison. Scale bar for panels A and B = 200 μm, and for panels B and C = 100 μm.


SL
PVN


A
B
C
D

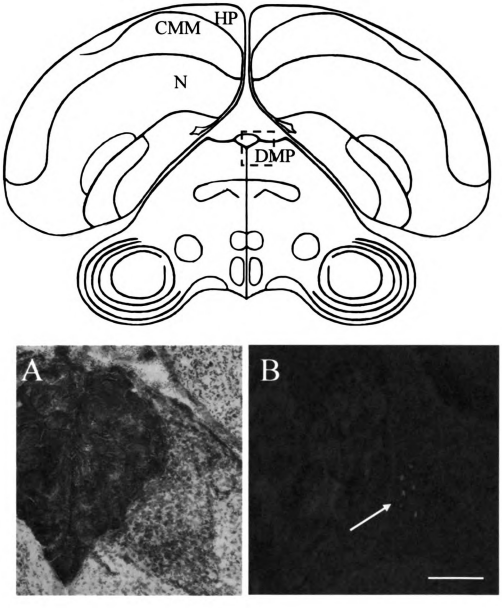


Figure 5.3. Coronal sections of a zebra finch brain at the level of the posterior portion of the dorsomedial thalamic nucleus (DMP; dashed red box). Fast blue injections into the dorsolateral subdivision of the hippocampus resulted in a few filled cells within the ipsilateral DMP (arrow) in males but not females. HP = hippocampus, CMM = caudomedial mesopallium; N = nidopallium. Scale bar = 100 μm.

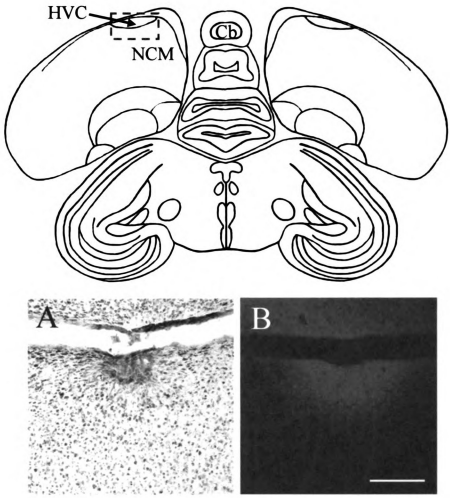
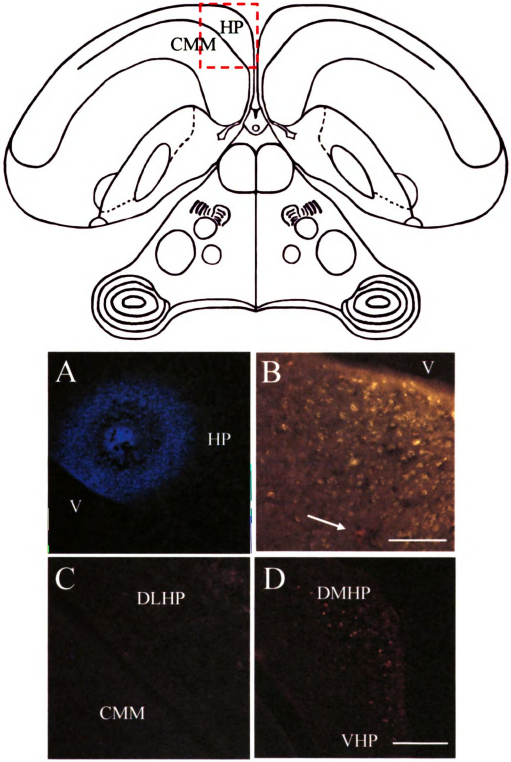


Figure 5.4. Coronal sections through a zebra finch brain at the level of HVC. Red, dashed box indicates the region depicted in the thionin stained (panel A) and fast blue labeled (panel B) sections. NCM = caudomedial nidopallium, Cb = cerebellum. Scale bar = 200 μm.

Figure 5.5. Iontophoretic injections of fast blue into the hippocampus (panel A) and fluorogold into the caudomedial mesopallium (CMM; panel B at arrow) of a male zebra finch resulted in fast blue filled cells in the CMM (panel C) and fluorogold labeled neurons in each of the hippocampal subdivisions (panels C and D). DLHP = dorsolateral subdivision, DMHP = dorsomedial, VHP = ventral, V = ventricle separating the hippocampus from CMM. Scale bar for panels A and B = 50 μm and for C and D = 200 μm.


HP
CMM
A
HP
V
B
V
C
DLHP
CMM
D
DMHP
VHP

DISCUSSION

The projections to the hippocampus detailed in this study indicate connections with nuclei involved in song behavior and, together with song-specific responses observed in the hippocampus (Chapters 1, 3 and 4, Bailey et al., 2002; Bailey & Wade, 2003), suggest a role for the region in responses to vocal communication signals or auditory-related memories in the zebra finch. Moreover, based on the present study and the one examining the efferent projections of the hippocampus by Székely & Krebs (1996), the zebra finch hippocampus appears to possess reciprocal connections with several of these regions that are directly or may indirectly be involved in song perception or memory, such as the CMM and SL in males and females and DMP in males.

Injections of a retrograde tracer into DLHP gave rise to only ipsilaterally labeled cells, which is similar to observations in zebra finches and pigeons by others who have examined the projections of this region using an anterograde tracer (Casini et al., 1986; Casini et al., 1986; Krayniak & Siegel, 1978; Székely & Krebs, 1996). For example, following anterograde tracer injections into the DLHP, terminating axons were consistently observed in the rostromedial medial striatum (MSt), preoptic regions, PVN, DMP, arcopallium, SL and the central gray (Székely & Krebs, 1996). In parids like the black-capped chickadee, the volume of the SL is larger than in a species that does not store food and increases in fall when the bird is exhibiting peak caching behavior (Shiflett et al., 2002). The septum may therefore be involved in spatial or relational memory along with the hippocampus, and in the zebra finch a relationship may exist between the regions that results in the processing of memory for relevant auditory signals in the form of a "contextual song memory" mentioned above. In a non-songbird, the

intertelencephalic connectivity of the hippocampus is quite similar. Ipsilaterally, the hippocampus in homing pigeons has reciprocal connections with the SL and Tn, and a contralateral projection is observed from the hippocampus to the SL and MSt (Atoji et al., 2002).

The interconnectivity of subdivisions within the zebra finch hippocampus also parallels that from a non-songbird. DLHP projects to both the DMHP and VHP (Székely & Krebs, 1996), and retrogradely-labeled cells in these regions were consistently detected in the present study in both males and females. In the pigeon, a more detailed analysis of intrahippocampal connectivity has been performed by stimulating individual regions of the hippocampus and recording evoked field potentials from neighboring subdivisions. Based on the cytoarchitecture of the region, the pigeon hippocampus also has dorsolateral, dorsomedial and ventral subdivisions that are further subdivided and connected in a manner similar to that observed in the zebra finch (Hough II et al., 2002). For example, the dorsal DLHP projects to the dorsomedial subdivision, which send axons that synapse in the medial VHP, which ultimately project back to the DLHP to its ventral portion where axons of that region exit the hippocampus. These networks of connections suggest a similarity to the trisynaptic pathway (Amaral & Witter, 1989; Eichenbaum et al., 1996) in the mammalian hippocampus. However, the topology of the region present in mammals, dentate gyrus to Ammon's horn to subicular and entorhinal cortices, appears to be different from that present in birds. If one presumes that adjacent parts of the dorsal cortex are homologous to the entorhinal and subicular cortices (Hough II et al., 2002; Butler and Hodos, 1996), then the topological order in zebra finches would be Ammon's horn ("hippocampus proper," or the subdivisions described above) to dentate gyrus (the

parahippocampal area as described in pigeons) to subicular and entorhinal cortices (perhaps SPf; Székely & Krebs, 1996; Butler and Hodos, 1996).

The primary differences between this study and that by Székely & Krebs (1996) are my observations of retrogradely labeled cells in the CMM and HVC. In their work, hippocampal projections observed are only in rostral portions of the mesopallium just dorsal to the most lateral extent of the CMM where the lateral ventricles terminate (Székely & Krebs, 1996), whereas in the present study, retrogradely-labeled cells were detected in CMM from its border with the lateral ventricle to the medial extent of the region. Although this difference may be based on the uptake or kinetics of the different tracers, the hippocampus in mammals receives substantial auditory input and the electrophysiological properties of neurons within the hippocampus can be modulated by lesion or stimulation of those regions. For example, in rats, sensory evoked activity in the hippocampus is modulated by damage to the entorhinal cortex or medial septum (Foster, Hampson, West, & Deadwyler, 1988), and in rabbits, auditory stimuli are more potent than visual stimuli in their ability to modulate electrophysiological properties of dorsal hippocampal neurons (Astikainen, Ruusuvirta, & Korhonen, 2005). In zebra finches, cells in the CMM appear to be the primary means for conveying auditory information to the hippocampus, and the two structures may be part of a circuit that is integral to the consolidation of song-related memories.

The sexually dimorphic projections observed in the present study are intriguing also, although more birds may be needed to confirm statistically that the projections differ between the sexes. Although the efferent connections of the hippocampus were previously examined in both sexes (Székely & Krebs, 1996), whether sex differences

were uncovered was not reported. In the present study, in males but not females, the hippocampus appears to have reciprocal connections with DMP, a thalamic nucleus that projects to the left and right medial portions of the magnocellular nuclei of the nidopallium (mMAN), which project ipsilaterally to the motor nucleus HVC (see Chapter 1; Vates et al., 1997). Further, the downstream motor nucleus RA projects to DMP, creating a "thalamo-cortical" circuit that is involved in the coordination of vocal signals (Vates et al., 1997). That the hippocampus has reciprocal connections with DMP suggests perhaps a modulatory role for hippocampal neurons on the function of cells within DMP. In addition, in three out of four males and in only one out of four females were labeled cells observed in HVC following hippocampal injections of the retrograde tracer, although this difference may be due to the dramatic difference in size between the male and female HVC. In female but not male zebra finches, retrogradely-labeled neurons were found in the PVN, suggesting a sexually dimorphic role for the region that may or may not be related to song behavior. However, in male European starlings, axons from another hypothalamic nucleus, the medial preoptic nucleus, terminate in a region of the arcopallium surrounding RA, suggesting an indirect pathway through which the region may influence song behavior and specifically motivation to sing (Riters & Alger, 2004).

The hippocampus in zebra finches has several intratelencephalic connections that provide the region access to auditory information and suggest a modulatory function for the structure in the processing of or response to song stimuli. For example, in adult females, immediate early gene activity in response to conspecific and heterospecific song is observed in CMM, but only conspecific songs induce immediate early gene activity

within the hippocampus (Bailey et al., 2002). Although further examination of the neural loop between these regions is necessary, these data collectively point to a means by which zebra finches may be able to form memories relating particular, relevant songs to specific environments (see Chapter 1) or perhaps distinguish other individual conspecifics. The questions that remain are the specific nature of these connections, particularly the sexually dimorphic ones, when they develop, and how they are regulated. The answers will be important to further our understanding of the nature of vocal communication and the continuous neural modifications that may influence the sender-receiver relationship.

CHAPTER SIX

Effects of lesions of the hippocampus in adult and juvenile male and female zebra finches on song-related behavior

INTRODUCTION

Responses to conspecific song have been detected in the hippocampus using a number of different neurochemical and behavioral paradigms. I found that in adult female zebra finches conspecific song presentations produce a higher density of FOS-immunoreactive neurons in the hippocampus than heterospecific songs, randomly generated tones or no songs (Bailey et al., 2002). In juvenile females at d30, FOS-positive cells are also highest in the hippocampus following conspecific song exposures compared to heterospecific songs, tones or silence (Bailey & Wade, 2003). In d45 birds, FOS and ZENK immunoreactivity are highest (but not significantly higher) in the hippocampus after conspecific song presentations compared to heterospecific songs and silence (Bailey & Wade, 2005). Several other studies have reported immediate early gene activation in the hippocampus in response to auditory stimulation with novel conspecific songs and/or tutor song (Bolhuis et al., 2001; Bolhuis et al., 2000; Eda-Fujiwara et al., 2003; Kimpo & Doupe, 1997) and after pairing of conspecific song with a discrete visual stimulus (Kruse et al., 2004). Male zebra finches presented with conspecific song stimuli showed a 40% increase in phosphorylated extracellular-signal regulated kinase (pERK) in the hippocampus compared to a silent control (Cheng & Clayton, 2004); pERK activation is integral to induction of the ZENK gene. Additionally, neural activity in the hippocampus of male and female zebra finches measured using optical imaging following the injection of a voltage sensitive dye was higher during than before and after conspecific song presentations (Kakeue, Kaminosono, Kitamura, Ogawa,

& Oka, 2004). However, other studies have stated explicitly that no immediate early genes are observed in the hippocampus following song presentations (Mello & Clayton, 1994; Stripling et al., 2001), and another normalized staining in the NCM to the overlying hippocampus (Jin & Clayton, 1997), suggesting minimal or baseline expression within the region. Although it was reported that electrophysiological responses to male zebra finch song are not found in neurons within the hippocampus (Chew et al., 1995), auditory responses have been observed in some hippocampal cells using conspecific song and white noise as stimuli (Dr. Mark Hauber, personal communication).

The telencephalic connections of the zebra finch hippocampus detailed in Chapter 5 suggest a potential role for the region in song-related behavior. For example, in males and females, the hippocampus appears to have reciprocal connections with the auditory area CMM and the lateral septum, a region known to be involved in motivated behavior in birds (see Chapter 5). The hippocampus in males also projects to and receives efferents from DMP, a thalamic nucleus part of a circuit involved in song production. That neurons within the hippocampus receive information from regions involved in song perception, motivation and production and that the hippocampus projects *back to* those areas suggests a potential modulatory role for the hippocampus in specific song-related behaviors.

It is clear that the hippocampus in some cases responds to conspecific song and that intertelencephalic connections exist that provide song-related information to the region, but the specific nature and purpose of these responses and connections have not been examined. Conspecific song induced activation within the hippocampus suggests a relationship between auditory perception and song memory, potentially involving (1) the

consolidation, retrieval or maintenance of a song template, and/or (2) the encoding of information about the environment in which song was heard, consistent with the relational memory function of the structure observed in a multitude of studies using avian and mammalian species (Colombo and Broadbent, 2000; Eichenbaum, 2000; Squire, 1992). Given the role of the hippocampus in specific types of memory formation (Oberlander, Schlinger, Clayton, & Saldanha, 2004; Patel et al., 1997a; Watanabe & Bischof, 2004), it may be involved in the development or utilization of song memories in a manner consistent with one of or both of the two hypotheses listed above.

The following experiment was designed to determine the effects of lesions to the hippocampus during development or adulthood on song-related behavior in the zebra finch. Following a lesion or sham control, birds were given tests of song production (males), two tests of song perception (tutor's song versus that of another conspecific, and conspecific versus heterospecific song; both males and females), and spatial memory (both sexes) as a control. If, for example, the hippocampus is important in adult song perception or production, then destruction of the structure in mature birds should result in impaired performance. This study also tested the possibility that the hippocampus is involved in the song learning process. Decrements in song production or song perception following lesions of the structure at d20 or d45 in males and females, just as they enter the template formation and sensorimotor integration phases (at least as defined in males; Nordeen & Nordeen, 1997), respectively, would indicate an integral role for cells within the hippocampus in the development and adult expression of normal song behavior.

MATERIALS AND METHODS

Animals, Surgery and Housing

To confirm the validity of the behavioral measures used in this experiment, 9 adult male (5 hippocampal lesion and 4 sham control, see below) and 8 intact adult female zebra finches were obtained from group cages in which birds of the same sex were housed together. They were in visual and auditory but not physical contact with the other sex. All of the females were from Magnolia Bird Farm (Riverside, CA) and were in their home aviaries at least two weeks prior to testing. Eighty-nine birds from the breeding colony were used in the actual experiment. Of those, 15 died following surgery or shortly thereafter, 12 had lesions ventral to the hippocampus, and three (one female lesioned at d20 and two males lesioned or sham-lesioned in adulthood) did not eat at all from the baited cup during the spatial memory test (see details on the test below) and were removed from the experiment. The number of birds remaining for the groups was as follows: d20, n = 10 males and 9 females; d45, n = 10 males and 10 females; and d100, n = 10 males and 10 females.

All birds were maintained on a 12:12 light:dark cycle (lights on at 0700). While housed in the communal aviaries, animals were provided with *ad libitum* access to seed, water, gravel, and once-weekly supplements of oranges, spinach, or hard boiled chicken eggs and bread. Birds in each group were removed from their communal aviaries at d100 and placed in individual cages (11" x 9" x 12"; see rationale below) with free access to water, gravel, and seed (except before spatial memory test, see below). While in individual cages, birds continued to have visual and auditory contact with other males and females.

Birds were deeply anesthetized with isoflurane and bilateral injections of 0.1 μl of 10 mg/ml ibotenic acid (ICN Biomedicals, Costa Mesa, CA; dissolved in 0.1 M phosphate buffered saline (PBS), pH 7.4; lesion group) or 0.1 μl PBS (sham) were made into the hippocampus using coordinates and procedures described in the experiment in Chapter 4, determined through pilot work, and aimed only at the dorsolateral subdivision of the structure where I detected conspecific song-induced immediate early gene activation (Chapters 2 and 3; Bailey et al., 2002; Bailey & Wade, 2003, 2005). In d45 and adult birds, holes were drilled through the skull and the micropipette was lowered 0.3 mm relative to the dura. The skull of d20 zebra finches is not fully ossified, so no holes were drilled, and the micropipettes easily pierced through the skull's surface. The ventral measurement for birds of this age was 0.55 mm relative to the surface of the skull to account for the 250-300 μm thickness. Since zebra finches need parental care for feeding until approximately d35, and because song learning in the presence of a tutor begins around d25 and continues through approximately d65, juveniles were returned to their home cages following surgery and recovery. Birds were returned only after I determined that the effects of the isoflurane had dissipated, mainly by ensuring that locomotion was fluid and that birds (at least those d45 and adult) were able to balance on perches without trouble. Lesions to juvenile birds were performed near the end of the light cycle to lessen the number of encounters with other birds immediately following the surgery and to allow for additional, unimpeded recovery overnight.

Behavior Testing

All behavioral tests (except those for song production in adult males before the lesion, noted below) were done in the same room. Therefore, the spatial memory test was run first in all animals to avoid effects on performance in the task that may have been due to pre-exposure to the environment. The order of the remaining tests (two tests of song preference in males and females and "after lesion" song production tests in all males) were counterbalanced and all tests were separated by intervals of approximately 24 h.

Spatial Memory Test(s)

I designed a modified "t-maze" to examine spatial memory in zebra finches. The apparatus (see Figure 6.1), consisted of a wood frame and steel mesh on all sides through which behavior could be observed and recorded. This portion of the chamber was designed so the birds could clearly see the environment outside of it. At the base was a wood frame/steel mesh enclosed column that led vertically to left and right arms. Cut into the bottom of the column were four identical doors used as release points. In the apparatus were six identically colored and patterned cups with closable flaps, three in each arm, arranged in such a way that the arms looked identical at their entrance points. The chamber was situated in a room such that landmarks were easily identified based on their relation to the walls of the apparatus (a bookshelf, curtain, video camera, light above the center of the chamber, proximity to walls of the room, different shapes of the same color on adjacent walls, patterns of acoustic tile affixed to walls, etc.). The natural response of birds tested was to fly up the column, and the intent of the task is that they

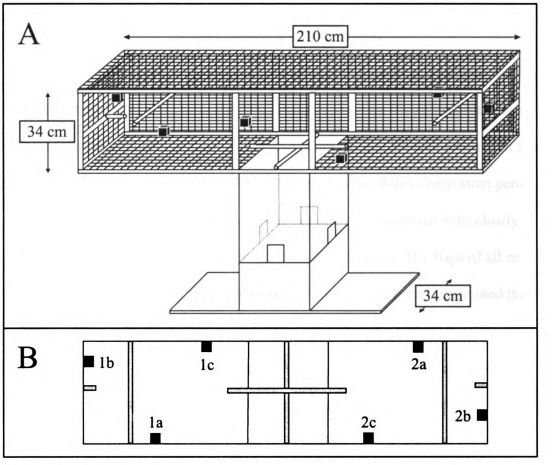


Figure 6.1. (A) Chamber used for spatial memory tests following hippocampal lesions, frontal view. Doors at the bottom of the chamber were used as entrance points. (B) Chamber diagrammed from above. Perches (indicated in grey) at the ends of the chamber that run parallel with the front of the maze sat 16 cm above the floor of the arms, and those that run perpendicular in each arm were 20 cm above. In the middle of the chamber, 3 cm above the floors of the arms, were perches in the shape of a plus, and the part of the plus that runs parallel with the front of the chamber extended 5 cm into each arm. The plastic cups (2 cm x 2 cm x 3 cm) were also at varying heights: cups 1a and 2a were 19 cm above the floor of the arms, cups 1b and 2b 15 cm, and cups 1c and 2c 2 cm. Perches were not placed directly below/adjacent to a cup so that birds would not land by one randomly. The perches were placed nearby and just above so that birds would only approach a cup to eat its contents.

entered and spent most of their time in the arm where seed was found previously, even from random release points, based on spatial cues in the room.

Prior to testing, birds were individually housed without food for approximately 4 h to increase motivation to perform the task. Food deprivation began immediately after the onset of the light portion of their cycle, since zebra finches fill their crops with seed over the course of the morning hours (Zann, 1996). Following this deprivation period, each bird underwent "acquisition" trials, which began by placing them individually into the chamber through the front door in the bottom of the column. The flaps of all cups were open, and only one of the cups was filled with seed. Pilot work determined that almost all birds took 20 min or less to acclimate to the chamber and eat from the baited cup. Thus, birds were given 20 min (maximum latency) to explore the chamber and locate the seed. Once the seed was found (or if 20 min had passed), each bird was given 30 sec to eat, after which it was returned to its individual cage in a separate room. Every 10 min, birds were placed back into the chamber through the same door until the arm that contained food was entered within 30 sec on three consecutive trials (criterion). Upon reaching criterion, birds were returned to their individual cage for one hr.

The cups that did not contain seed were filled and then emptied to eliminate any olfactory cues birds may use to locate the seed, although the only avian species that appear to rely heavily on olfactory cues are those that migrate or are carnivorous (Malakoff, 1999). Also, zebra finches forage for and choose seeds based mainly on visual cues like size and color to limit time spent foraging (Zann, 1996). Following the one hr interval upon reaching criterion, birds underwent four "probe" trials, each separated by 10 min, which tested their memory for the food location. Each bird was

placed through each of the four doors of the chamber at random and arm entries, time spent in each arm, and the number of cups examined or flaps lifted were measured. A cup was noted as "examined" whenever a bird leaned from an adjacent perch toward the cup or flew from a perch to the steel mesh next to a cup. Cups closest to the floors of the arms of the chamber could be examined by a bird that hopped along the floor to them. A bird was given 30 sec to eat if it found the seed by lifting the flap of a specific cup. Birds were removed from the chamber if seed was not eaten within 20 min.

For the males used to pilot the spatial memory task, initial latencies and the number of trials to reach criterion were compared by means of unpaired t-tests. For the experimental birds, a mixed model analysis of variance (ANOVA) was used to determine whether differences existed in these measures by treatment group, age and sex. A mixed model analysis of variance was used to determine differences in the latencies to find the food between the hippocampal- and sham-lesioned animals in the subsequent testing sessions, the change in the latencies over time, the number of incorrect cups examined or flaps lifted during those trials, and any potential interactions.

Song Preference Tests

Each zebra finch was placed in the chamber described in Figure 2.2. In order to determine preference for one song over another, songs, burned onto compact disc and played via a Sony CD Walkman (#D-E220), were broadcast synchronously from speakers at each end of the apparatus ("approach zones"; similar to procedures used by Clayton, 1988 and Miller, 1979b). The middle of the apparatus between the two ends was considered a "neutral zone" (Clayton, 1988). Birds were placed in the chamber and

allowed to acclimate for 1 hour. Then, the songs of two zebra finch males (one from the father and a dissimilar, novel conspecific song chosen randomly from a bank of six) or those of a conspecific and heterospecific (both novel and randomly chosen from banks of six) were played simultaneously for 20 min. For each bird's first preference test, the song that should be preferred (father's song over that of another conspecific, and conspecific over heterospecific song) was played from the speaker on the side of the room opposite that where the food was located in the spatial memory test (see below) to eliminate the preference for a "zone" of the chamber that may be conferred by memory for the food location. Following song delivery, birds were removed from the chamber and returned to their individual cages. Behavior was recorded with a Sony Digital Handycam (model DCR-TRV230) and uploaded to a PC as described above.

In nature, zebra finches respond to song with a suite of behaviors. Females, for example, will fly to and from a perch several times before beginning to "hop" from perch to perch near the song of a male, and will produce "tet" and distance calls in a male's presence (Zann, 1996). In the laboratory, tests of song recognition and preference have measured the amount of time a bird spends near a speaker broadcasting a particular song. Previous studies using this paradigm have described song discrimination as spending twice as much time in one approach zone than the other for a significant portion of the trial (Clayton, 1988). In pilot work described below, I also measured activity during song exposure by recording the number of calls and movement (turns on a perch and jumps from perch to perch) made in each zone of the chamber. Difference scores for each measure (responses to father minus other conspecific and zebra finch song minus heterospecific song) were obtained and analyzed between the sham and lesion groups

within each treatment age and sex by ANOVA. For the 8 females used to pilot this procedure, differences scores for each measure were compared using one sample t-tests.

Male Song Learning and Production

The father of each subject was determined by observing the male that entered a particular nest box consistently over a number of days. Fathers were temporarily removed and their songs recorded in the presence of a novel female. These songs were later used as indicated below (1) to compare to the songs of their male offspring to determine the influence of hippocampal lesions on song learning, and (2) as one of two simultaneously played conspecific songs in tests of song preference. All songs were recorded via an Optimus boundary microphone (Radio Shack) connected to a Sony Digital Handycam (model DCR-TRV230) and uploaded to a PC as Windows Media Video files using Windows Movie Maker (version 2.1.4026.0). Each .wmv file was converted to Audio Video Interleave format using RiverPast Video Cleaner Lite (version 6.5.0.50717). Audio was extracted from the .avi files using Adobe Audition (version 1.0) and background noise was reduced in fathers' songs only (since only they were used for tests of song preference) using a Fast Fourier Transform size of 512 points. All song files were saved as Windows PCM (.wav) with a sample rate of 44,100 Hz and a resolution of 16 bits.

All song was recorded when birds were adults. To determine whether lesions of the hippocampus affect song production or stability in adulthood, songs pre-lesion (and pre-sham) were recorded from adult males by placing each in a cage with a novel female. Most males sang within 20 min following the introduction of a female, so to ensure equal

experience between the sexes, females were placed in the same room in a cage with a novel male for 20 min before and after the lesion/sham. Following the lesion (after approximately 9 days; range = 7 to 10 days), adult males were again tested for song production in the presence of a female. Over development, the songs of male zebra finches become almost identical to those of their tutor, usually their father (Immelmann, 1969). To determine whether lesions of the hippocampus affect the matching of tutor song, the songs of males lesioned as juveniles were recorded in adulthood in the presence of a novel female. The songs of males lesioned at d20 were recorded at an average age of approximately 152 days post-hatch, and those lesioned at d45 at d148. Females lesioned or sham-lesioned as juveniles were put in a cage with a novel male once for 20 min.

The comparison of songs between tutor and pupil or pre- and post-manipulation has been a longstanding means of determining the similarity of songs and/or whether a particular neural or behavioral manipulation impacts song learning, production or stability (Jones, ten Cate, & Bijleveld, 2001). Zebra finch song consists of syllables or "notes" that, when viewed as a sonagram (see example in Figure 6.2), appear as distinct frequency traces separated by intervals of silence or with abrupt changes in amplitude (Zann, 1996). The order of these notes, including the intervals of silence between them, are grouped and repeated in "phrases" or "motifs" that in total last roughly 3-4 sec, resulting in a "song." Song comparisons made by human observers involve the examination of qualities of song such as the types, order and frequencies of notes as well as the length of silent intervals. While a basic visual assessment of song is necessary

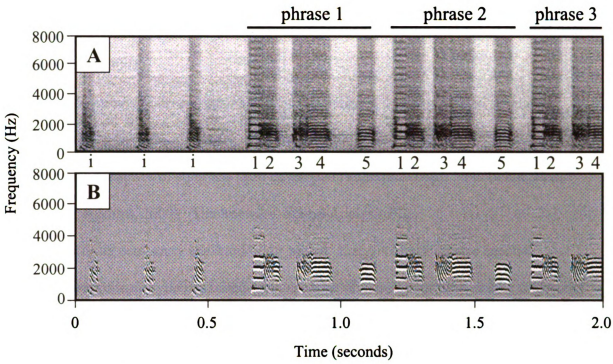


Figure 6.2. Sonagram (A; frequency over time) and spectral derivative (B; change in power over time) of the same song portion from a male zebra finch. Introductory (i) and individual notes (identified as 1-5) are indicated. Zebra finch song consists of introductory notes that are followed by notes of particular frequencies and changes in amplitude that are repeated in a fixed order (a "phrase;" three phrases indicated). Note the "static" appearance of the background in the sonagram (A) and the elimination of that extraneous noise in the spectral derivative (B).

(and is used as part of the procedure in this experiment; see below), individual observers may differ considerably in determining which elements of song match and to what degree (Jones et al., 2001).

To eliminate the subjective bias or inexperience that may lead to unreliable determinations of song similarity, an automated procedure for measuring and comparing the acoustic features of song has been developed (Tchernichovski, Nottebohm, Ho, Pesaran, & Mitra, 2000). The freeware, Sound Analysis Pro (http://ofer.sci.ccny.cuny.edu/html/body_sound_analysis.html), creates spectral derivatives of song syllables based on the change of power over time, unlike traditional sonagrams that represent the power of sound as a change in frequency over time. Because of this, background noise is omitted almost entirely, and syllables and the phrases they comprise are more visually distinct (Figure 6.2B).

Similarity scores of "pupil" versus "tutor" songs (for birds lesioned as juveniles) and songs "before" versus "after" lesion (for birds lesioned as adults) were obtained as follows. First, in Sound Analysis Pro, adjustments were made to each song based on guidelines detailed in the user's manual (Tchernichovski, Swigger, & Mitra, 2004). The amplitude thresholds of songs were calibrated by adjusting an unscaled dB sound level within a frequency range of 860-8600 Hz. For each song, amplitude was adjusted to eliminate extraneous points in the tracing that were clearly not associated with a syllable, and Wiener entropy, a measure of tonal versus harsh sounds, was adjusted to eliminate low amplitude background noises. Similarity scores were then calculated, with one phrase from each song used for comparison (see below). The overall similarity score calculated by Sound Analysis Pro is an aggregate of three values: (1) percent similarity,

the percentage of a tutor's sounds (individual note types) present in the song of the pupil or between a bird's song before and after a manipulation; (2) mean accuracy, the spectral characteristics of those individual notes; and (3) sequential match, a comparison of the temporal order of the notes, their duration, and the length of silent intervals between them. Pupil versus tutor songs were compared asymmetrically (Figure 6.3; used when one song is a model for another; Tchernichovski et al., 2004), and the songs of birds before and after hippocampal lesion were compared symmetrically (Figure 6.4; used when one song is not categorized as a model for another).

All comparisons were done blind to treatment condition. Unpaired t-tests were used to determine whether similarity measurements in birds lesioned as adults differed significantly from the adult sham-lesioned controls. In addition, the sonagrams of adult birds before and after lesion were qualitatively examined to determine if notes were added or subtracted, and whether the order of the notes remained consistent following the lesion. Similarity measurements for birds lesioned at d20 and d45 were analyzed using a factorial ANOVA for age and treatment.

Tissue Collection

Following behavior testing, animals were given an overdose of Equithesin and perfused with 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Brains were post-fixed for one hour in paraformaldehyde, sunk overnight in 30% sucrose in PBS, cut frozen on a cryostat into 30 μm coronal sections onto Superfrost Plus slides (Fisher) and thionin stained as described in Chapter 5. The hippocampus of each lesioned bird was reconstructed using Neurolucida software (version 6.02.1,

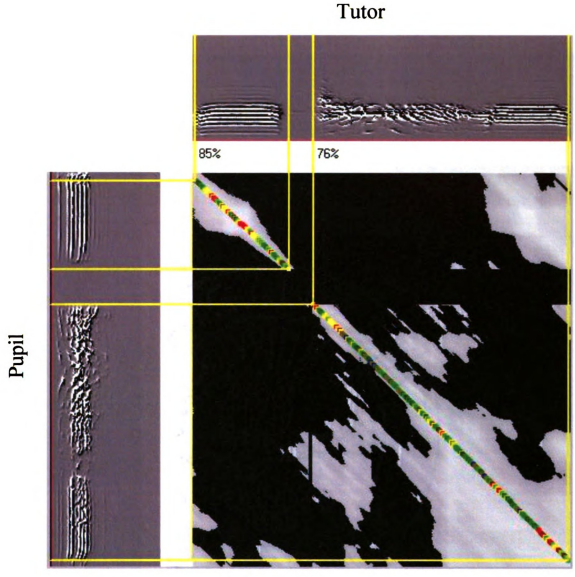


Figure 6.3. Example of an asymmetric similarity matrix obtained by comparing a phrase from the song of a male zebra finch (tutor) and another from the song of one of his male offspring (pupil). Yellow lines indicate the boundaries of individual notes or phrases (and are used to obtain the "percent sequential match" score). High similarity values are restricted to the diagonal, indicating that the individual notes in the tutor song were learned and matched by the pupil in sequential order. Warmer colors along the diagonal line indicate higher similarity ("percent similarity"). "Mean accuracy" measures the power of the individual notes on a scale from white (high match) to gray to black (no sound or no match).

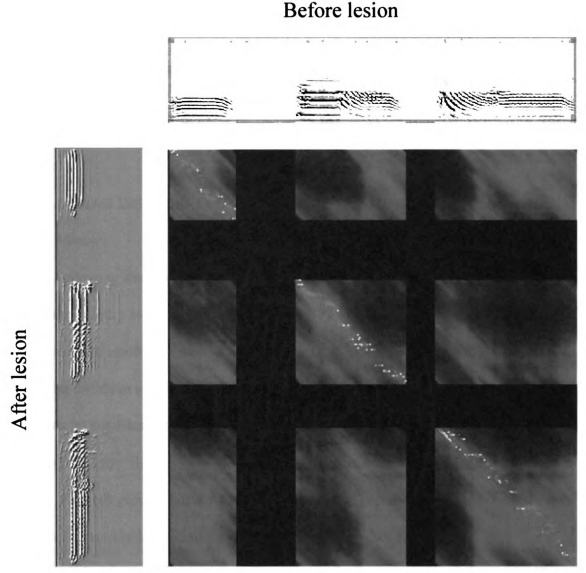


Figure 6.4. Example of a symmetric similarity matrix obtained by comparing a phrase from the song of a male zebra finch before and after a lesion of the hippocampus. High similarity values are restricted to the diagonal, indicating that the individual notes in the song pre-and post-lesion are in sequential order, and the warmer colors along the diagonal line indicate higher similarity ("percent similarity"). Overall, "mean accuracy" between the individual notes (indicated by gray and white colors) and the temporal order of the notes ("sequential match;" black bars) are high.

MicroBrightField, Inc.). Contours were traced in every fourth section around the border of the hippocampus and the area within the structure in which cell bodies were destroyed to confirm the accuracy and extent of the hippocampal lesion. Total hippocampal and lesion volumes were computed using NeuroExplorer (version 4.01.1, MicroBrightField, Inc.) to determine the percent of the structure ablated in each animal injected with ibotenic acid.

RESULTS AND DISCUSSION OF VALIDATION OF METHODS

Spatial Memory

Following the hippocampus or sham lesion, males did not significantly differ in their initial latency to eat from the baited cup (t (7) = 0.90, p = 0.398) or the number of trials needed to reach the criterion level (t (7) = 0.14, p = 0.894; Figure 6.5). Analyzing across the last three acquisition trials (during the last of which, criterion was reached), there was no significant difference in latency to eat from the baited cup between the groups (F (1, 14) = 0.73, p = 0.422) nor any interaction with time (F (2, 14) = 0.65, p = 0.537). For both groups, latency to eat from the baited cup decreased over the last three trials and approached significance (F (2, 14) = 3.49, p = 0.059).

Latency to eat from the covered, baited cup in the first probe trial was 600.25 +/- 99.73 sec in sham-lesioned males, an approximately 50% increase from the latency to eat from the opened cup during the last acquisition trial (t (3) = 2.73, p = 0.072). Hippocampal-lesioned males had an average latency 961.6 +/-112.50 sec in the first probe trial, up approximately 60% from the last acquisition trial (t (4) = 2.70, p = 0.054). No significant difference in latencies were seen between the treatment groups over the

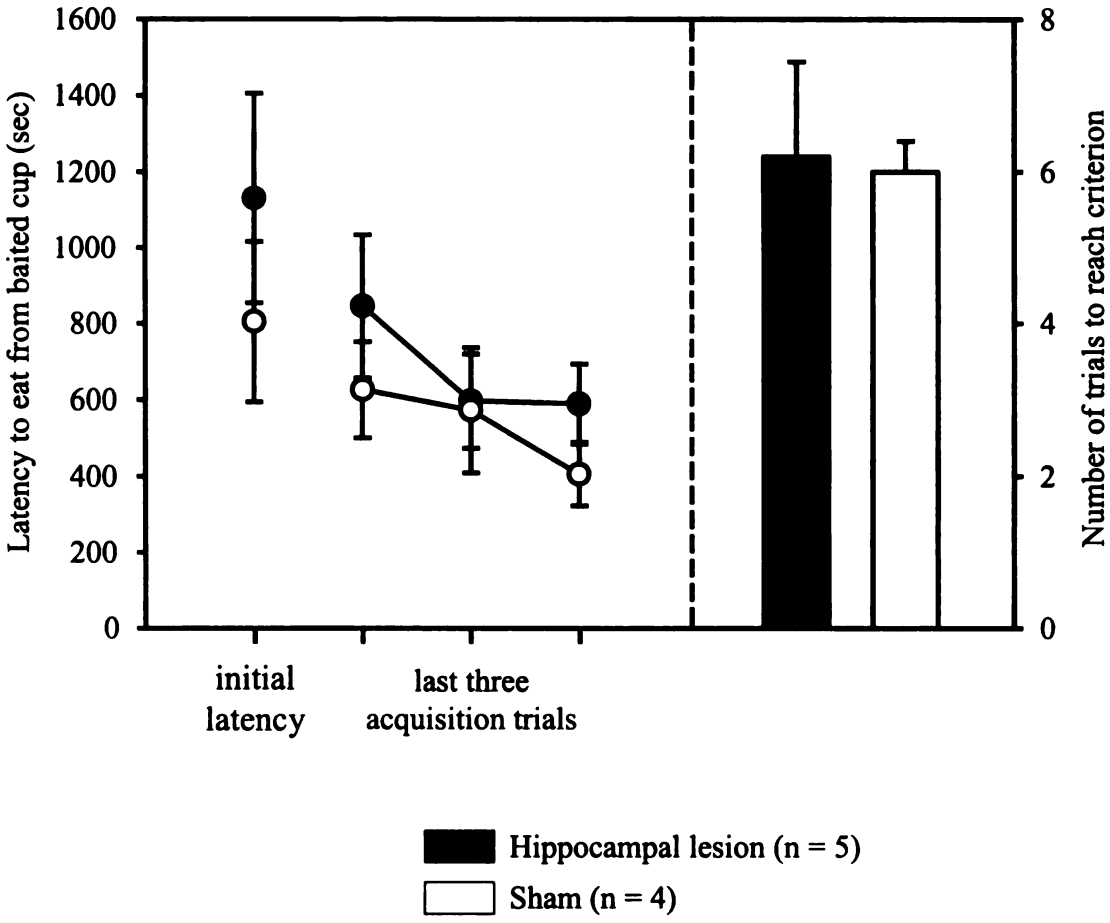


Figure 6.5. Performance during the acquisition phase of a spatial memory test by five hippocampal-lesioned and four intact male zebra finches. Initial latency (sec) to eat from the baited cup by the food deprived birds upon first exposure to the chamber, the average time needed by each group during the last three acquisition trials, and the average number of trials needed to reach criterion are indicated. Latencies of both groups of birds decreased significantly over the last three acquisition trials. No other significant main effects or interactions were found.

four probe trials (F (1, 21) = 2.97, p = 0.129), but there were differences in their patterns across the trials. Both groups showed a decrease in latency to eat from the baited cup from trial one to four (F (3, 21) = 12.14, $p < 0.0001$). The decrease in average latencies by the lesioned birds was steeper (Figure 6.6), and a significant interaction between latencies and treatment was found (F (3, 21) = 3.27, p = 0.042).

Overall, sham-lesioned birds spent significantly more time in the goal arm in the probe trials than hippocampal-lesioned animals (Figure 6.6; F (1, 21) = 11.59, p = 0.011). No significant change in time spent in the goal arm occurred across the trials (F (3, 21) = 1.27, p = 0.312), and this factor did not interact significantly with treatment group (F (3, 21) = 0.87, p = 0.470). Hippocampal-lesioned birds examined significantly more incorrect cups or lifted the flaps of those cups than control birds over the four probe trials (Figure 6.7; F (1, 21) = 11.84, p = 0.011). Mistakes were made by only two control birds on the second probe trial. The mistakes by the lesioned birds decreased significantly over the probe trials, resulting in a significant trial effect (F (3, 21) = 3.31, p = 0.040). No significant interaction between treatment group and incorrect cups examined over the trials was found (F (3, 21) = 2.35, p = 0.101).

The hippocampus of each lesioned bird in this pilot work was reconstructed as detailed above and, on average, just over 11% (range: 4.1-18.5%) of the volume of the hippocampus in lesioned birds was destroyed by the excitotoxin. Simple regression was used to determine whether the amount of hippocampal damage correlated with any of the behavioral measures. No significant correlations were found when the percent of hippocampal lesion was compared with the initial latency to eat from the baited cup during acquisition, the number of trials to reach criterion, the difference in latency to eat

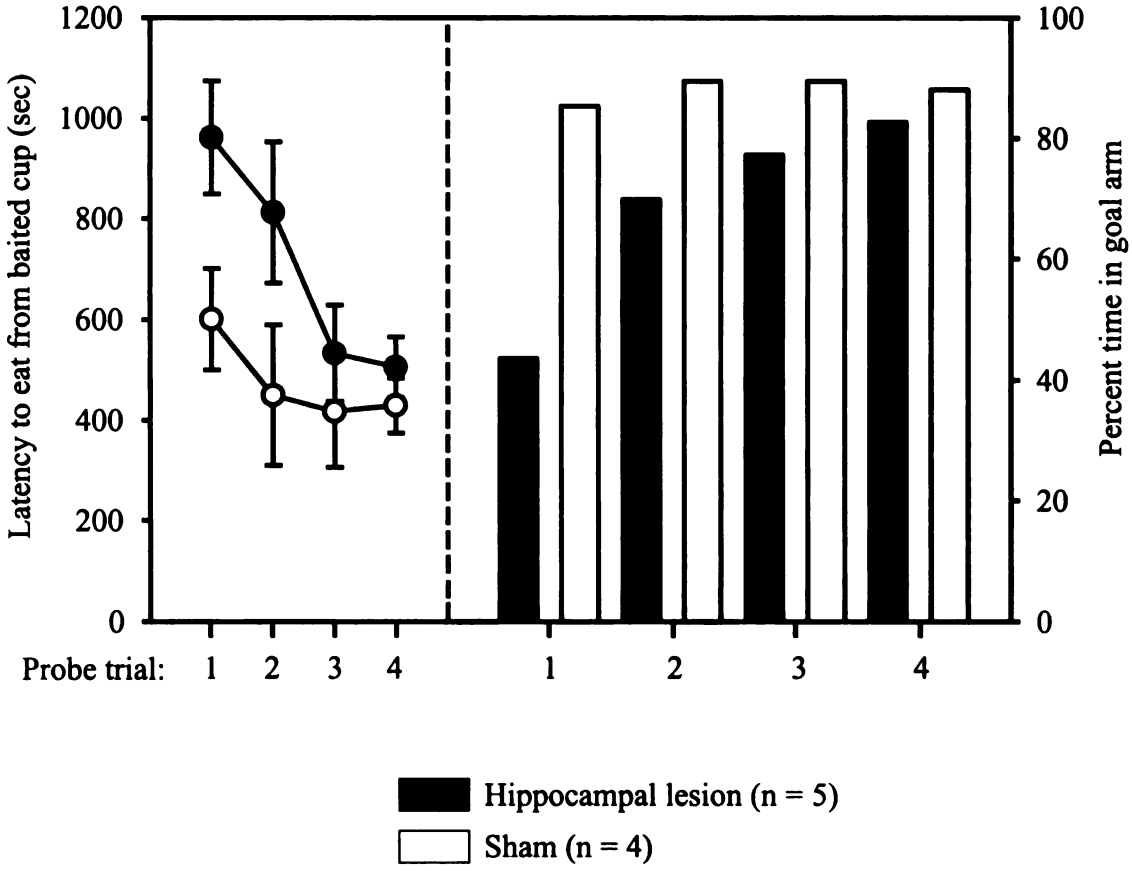


Figure 6.6. Average latencies to eat from the baited cup and percent of total time in the chamber spent in the goal arm by hippocampal- and sham-lesioned male zebra finches over four probe trials. Sham-lesioned birds spent significantly more time in the goal arm over the four probe trials than lesioned birds.

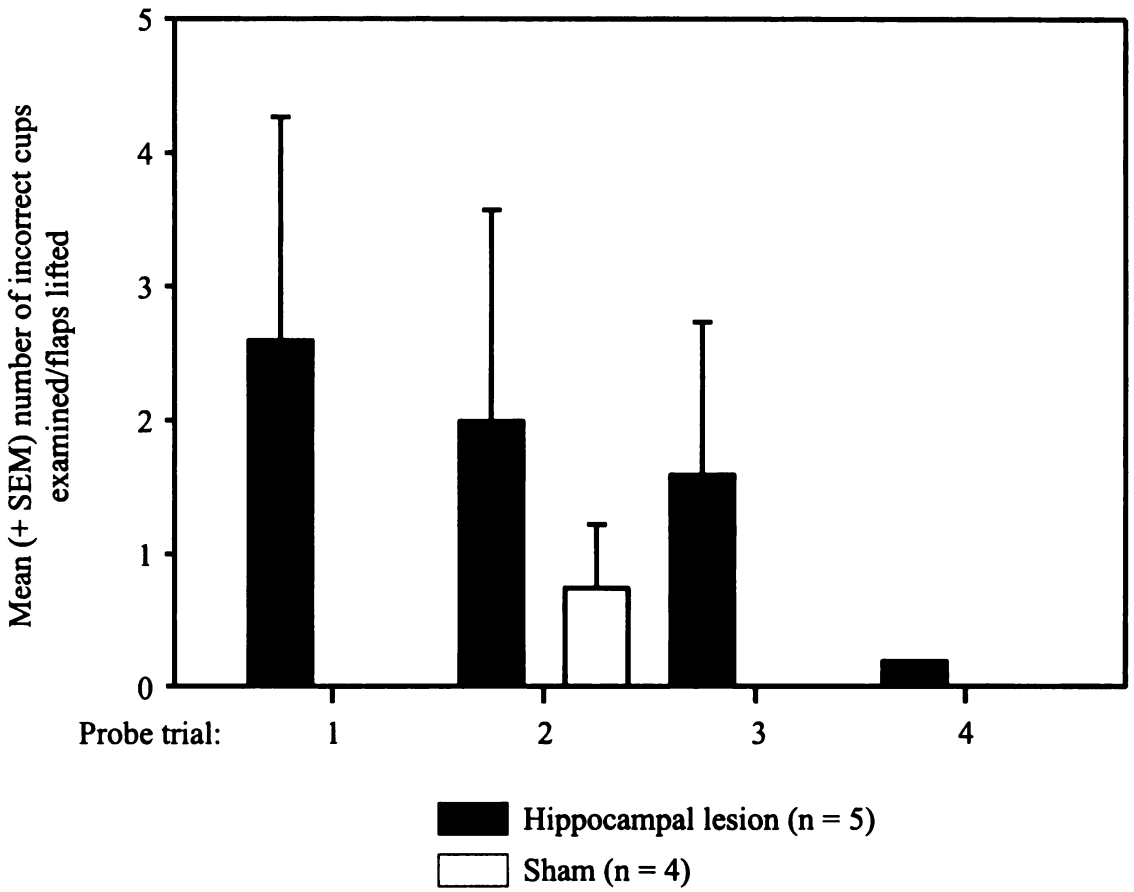


Figure 6.7. Mean (+ SEM) number of incorrect cups examined or flaps of those cups lifted by hippocampal- and sham-lesioned male zebra finches over the four probe trials. Hippocampal-lesioned birds examined significantly more incorrect cups or lifted the flaps of those cups than control birds.

from the baited cup between the last acquisition and first probe trials, the number of incorrect cups or flaps lifted in the first probe trial, the percent time in the goal arm during the first probe trial, or the latency to eat from the baited cup in the first probe trial (all $F < 8.52$, all $p > 0.062$).

To my knowledge, three published studies have examined the spatial memory capabilities of zebra finches. In two of these studies (Oberlander et al., 2004; Patel et al., 1997a), food-deprived adult zebra finches were trained to remember the location of a food source in a compartment covered by a cardboard flap. Other equally-sized compartments were also covered with flaps but contained no food. Male and female zebra finches with bilateral hippocampal lesions (Patel et al., 1997a) were inhibited in their retrieval of the food source location as indicated by the number of flaps lifted over compartments that did not contain food, and there was no sex difference in performance. In a separate study using a similar task, the performance of males treated with estradiol improved more rapidly than that of control-treated ones (Oberlander et al., 2004). Another study examined the ability of hippocampal-lesioned adult males to find previously located seed in one of four feeders on the floor of an experimental chamber (Watanabe & Bischof, 2004). Lesioned birds made more visits than sham-lesioned controls to feeders that did not contain seed. However, lesioned males did not significantly differ from controls in their latency to find the seed, suggesting use of an alternative, non-spatial strategy.

Zebra finches, however, do not cache food, and therefore these "window shopping" tasks, commonly used with food-storers such as those in the parid and corvid families (Capaldi, Robinson, & Fahrbach, 1999; Krebs, Sherry, Healy, Perry, &

Vaccarino, 1989; Macphail, 2002), may not be suitable or relevant. Although zebra finches in the wild search for seed primarily on the ground, they prefer to remain perched when not foraging (Zann, 1996). The task I designed may more accurately reflect the natural behavior of the animals and eliminate stress associated with searching for seed on the ground (and that may also be compounded by the food deprivation).

The setup of the apparatus I designed allows for the recording of additional dependent variables besides the number of incorrect locations examined for a previously visited food source detailed in the studies above. Birds must choose one of two arms that via intra-maze cues are identical, but that can be differentiated based on the diverse extra-maze cues present. The arm entered upon placement into the base of the chamber is one indication of memory for a spatial location. However, an arm entered first may not be an accurate indicator of memory for the food source because I place the birds in the chamber and then leave the room; my movements and the sound of the testing room door closing cause birds to initially fly around within the chamber. This is why thirty seconds was chosen as the time for arm entry to reach criterion during the acquisition trials and whether the arm with the baited cup was entered "first" in the probe trials. After removal of distractions, birds make a choice to enter or remain in a particular arm, and given the length of food deprivation and a memory for the food source, correct arm entry for birds tested in the pilot study was within that timeframe. In addition to choosing the correct arm, the arm birds spend the most time in is also indicative of their memory for the food that was previously located in that arm. This type of variable cannot be measured in the experiments briefly described above (Oberlander et al., 2004; Patel et al., 1997a; Watanabe & Bischof, 2004) given the size and setup of the chambers. The number of

incorrect cups examined or flaps lifted in a specific arm of the chamber I designed may therefore be a more precise measure of spatial memory performance.

In this pilot work, lesioned males were clearly deficient in their ability to accurately locate the seed, but like hippocampal-ablated birds in another study (Watanabe & Bischof, 2004), they were, over time, able to find the seed in a manner consistent with the performance of the control birds, suggesting again some type of non-spatial strategy. Rats can switch from place learning dependent on the hippocampus to response learning dependent on the caudate nucleus (Packard & McGaugh, 1996). It is reasonable to suggest that a similar switch could occur in avian species as well. Importantly, the initial deficit in performance I show in hippocampal-lesioned birds, coupled with histology indicating cell loss, is proof that the structure is not functioning normally due to loss of the dorsolateral subdivision and that the apparatus is a reliable measure with which to detect the associated decrements in spatial memory behavior.

Song Preference

The amount of time spent or behaviors displayed in the side of the chamber playing heterospecific song was subtracted from that observed in the conspecific song side, and one sample t-tests were used to determine whether a preference existed for one side over another based on a hypothesized difference of zero for the difference score. The amount of time females spent in the side that played conspecific song was approximately 20 times more than in the side of the chamber that played heterospecific songs ($t(7) = 17.81$, $p < 0.0001$). Other measures were greater on average in the conspecific side of the chamber, but only approached significance. On average, about six

times more calls were made in the conspecific song side of the chamber (t (7) = 2.06, p < 0.078). Approximately five times more turns on a perch were made in the side of conspecific song than the heterospecific song side (t (7) = 2.32, p = 0.053). Four and-a-half times more jumps from perch to perch were made in the conspecific side of the chamber (t (7) = 2.05, p = 0.080). As in Chapter 1, calls and movement (turns and perch jumps) were summed and analyzed collectively. In the conspecific song side, about six times more calls and movement were made than in the heterospecific song side (t (7) = 2.17, p = 0.067). Finally, the number of entries birds made into each side of the chamber did not statistically differ (t (7) = 0.19, p = 0.852).

In the pilot work, females' preference for conspecific song was rather robust. Although only time spent in the side of conspecific song was significantly different from that in the heterospecific song side, other behavioral measures (calls, turns, perch jumps) were greatest in the zebra finch song side and approached significance, perhaps based on the small sample size. Other studies have shown that male and female zebra finches spend more time in the side of a chamber near conspecific compared to heterospecific song (Braaten & Reynolds, 1999; Lauay et al., 2004). The apparatus used for preference tests is similar to those used in prior studies (Clayton, 1988; Miller, 1979b) and the pilot data demonstrate its validity in the confirmation of a song preference.

Prior research using a number of different paradigms demonstrate that male (Adret, 1993; Clayton, 1990) and female (Clayton, 1988; Miller, 1979b; Riebel et al., 2002) zebra finches prefer their father's (tutor's) song to that of a novel conspecific or their own song (Adret, 1993). Given these findings, taken together with the robust preference by females for the conspecific over heterospecific song side of a chamber

detailed above, the test of preference for a father's song versus that of a novel conspecific was not piloted.

Song Production

As mentioned above, the song of a zebra finch typically consists of one phrase that is repeated several times. To ensure that the selection of only one phrase would be adequate in the determination of song similarity, multiple phrases from the songs of a tutor and his pupil and the songs of a male before and after lesion of the hippocampus were compared. Similarity measurements from the comparisons of four separate phrases from a tutor and pupil song are shown in Table 6.1. The percent similarity and mean accuracy were on average high between all the phrases examined. Percent sequential matches were low in four phrase comparisons due, I believe, to extraneous auditory stimuli and placement of the microphone. Since these males sang in the presence of a female, other sounds such as calls to the male by the female and courtship behaviors by both sexes (flying away from the perch and back, beak wipes against the perch or cage, tail quivers, dances on a perch, etc.; Zann, 1996) were picked up by the microphone. Also, it is possible that the microphone may not have picked up portions of a song depending on the direction a male was facing when singing. In the spectral analyses provided by the program, it is clear that in some phrases the power of individual notes is not as strong as the same notes in other phrases. These factors could have resulted in tracings that affected the power and sequential matches of particular song phrases determined and analyzed by Sound Analysis Pro.

Comparison Phrase					
Tutor	Pupil	Similarity Score	Percent Similarity	Percent Mean Accuracy	Percent Sequential Match
1	1	62.3	87	71.7	100
1	2	67.4	96	70.2	100
1	3	39.9	96	79.2	4.9
1	4	38.9	97	77.9	2.9
2	1	75.6	95	79.6	100
2	2	72.2	93	77.7	100
2	3	72.6	92	78.9	100
2	4	69.7	92	75.8	100
3	1	73.2	94	77.9	100
3	2	41.6	97	78	9.9
3	3	41	98	77.3	8.3
3	4	57.9	82	80.9	74.6
4	1	55.2	75	73.7	100
4	2	67.6	98	69	100
4	3	49.2	82	68.4	75.6
4	4	48.2	85	67.4	68.3
	MEAN:	58.3	91.2	75.2	71.5
	SEM:	13.5	6.9	4.5	40.2

Table 6.1. Similarity measurements obtained by comparing four different phrases (1-4) of a male zebra finch's song and those (1-4) of one of his male offspring. The "similarity score" is an aggregate of the three other values, which indicate parallels in the types of notes used (percent similarity), the frequencies of those notes (mean accuracy), and the lengths of silent intervals between the notes (sequential match). Means and standard errors for the measures are indicated at the ends of each column.

The same type of analysis was performed on three separate phrases from the songs of a zebra finch pre- and post-sham lesion (Table 6.2). Although one would expect the similarity between the song phrases from an unmanipulated bird to be very close to 100 percent, extraneous noise at the time of recording or proximity to the microphone when singing would affect song similarity as described above. In two out of nine comparisons, the percent similarity was less than the 90% or above desired for tests of self-similarity (Tchernichovski et al., 2004), in contrast to the range of 94-100 for the remaining seven comparisons. It is likely, again, that in these instances other auditory stimuli were factoring into the analysis.

The comparisons of multiple phrases in both types of song analyses (tutor vs. pupil and pre- vs. post-lesion) yielded generally similar results. For this reason, and due to the potential confounds mentioned above, only one phrase from each song type was used for comparison. A phrase was picked (1) if it was clear that no extraneous noises were part of it and (2) by the "sharpness" of its spectral derivative.

Given the overall equivalence in similarity measurements of individual phrases from the songs of a tutor and his pupil (Table 6.1) and those from a male before and following a lesion of the hippocampus (Table 6.2), Sound Analysis Pro appears to be a valid and reliable means of determining the relationship between song phrases. Also, similarity scores I obtained are equivalent to others that related tutor and pupil song phrases (Liu, Gardner, & Nottebohm, 2004) and examined the stability of adult song following lesion of a specific brain region (Coleman & Vu, 2005). The additional analysis comparing song phrases of adults pre- and post-lesion or sham by eye involves a straightforward, qualitative detection of note order. This method is standard in

Comparison Phrase					
Before	After	Similarity Score	Percent Similarity	Percent Mean Accuracy	Percent Sequential Match
1	1	87.2	97	91.6	97.7
1	2	65.2	83	83	95.3
1	3	89.1	96	93.8	98.5
2	1	87.9	98	92.1	97.5
2	2	65.5	83	82.8	94.9
2	3	90.9	97	95.2	98.1
3	1	95.9	100	96.7	99.3
3	2	81.4	94	89.1	97.2
3	3	95.2	99	96.1	99.9
	MEAN:	84.3	94.1	91.2	97.6
	SEM:	11.6	6.5	5.2	1.7

Table 6.2. Similarity measurements obtained by comparing three different phrases of a male zebra finch's song before and after a sham lesion of the hippocampus. The "similarity score" is a product of the three other values, which indicate parallels in the types of notes used (percent similarity), the frequencies of those notes (mean accuracy), and the lengths of silent intervals between the notes (sequential match). Means and standard errors (SEM) for the measures are indicated at the ends of each column.

determining song stability following a behavioral or physiological manipulation and is not open to subjective bias as are measurements of the specific frequency and temporal characteristics of song (Jones, ten Cate, & Bijleveld, 2001).

RESULTS – EXPERIMENTAL BIRDS

Histology

The amount of hippocampal damage produced by the excitotoxin ranged from approximately 10-23% for the birds used in this study (see Figure 6.8 for a sample bilateral hippocampus lesion). The effect of age of lesion on the amount of hippocampal damage observed in adulthood approached significance ($\underline{F}$ (2, 24) = 3.15, $\underline{p}$ = 0.061), with birds lesioned at d20 having the least damage, birds lesioned as adults the most, and those at d45 an amount intermediate between those two (Figure 6.9). There was no effect of sex ($\underline{F}$ (1, 24) = 0.03, $\underline{p}$ = 0.869) or interaction between age and sex ($\underline{F}$ (2, 24) = 0.36, $\underline{p}$ = 0.704) on the percent damage to the hippocampus.

Spatial Memory

Birds with hippocampal lesions did not significantly differ from their sham controls in their initial latencies to eat from the uncovered, baited cup during the first acquisition trial (all $\underline{F} < 1.61$, all $\underline{p} > 0.211$ for main effects of age, sex, treatment group and interactions) or the number of trials needed to reach the criterion level during acquisition (all $\underline{F} < 1.74$, all $\underline{p} > 0.187$; data not shown).

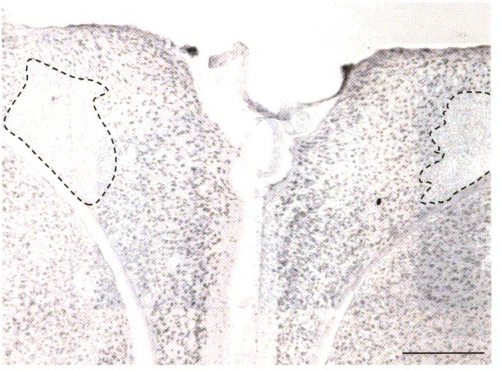


Figure 6.8. Coronal section through an adult male zebra finch brain at the level of the hippocampus detailing areas of cell death (traced by dashed lines) produced by injection of ibotenic acid at d20. Scale bar = 200 µm.

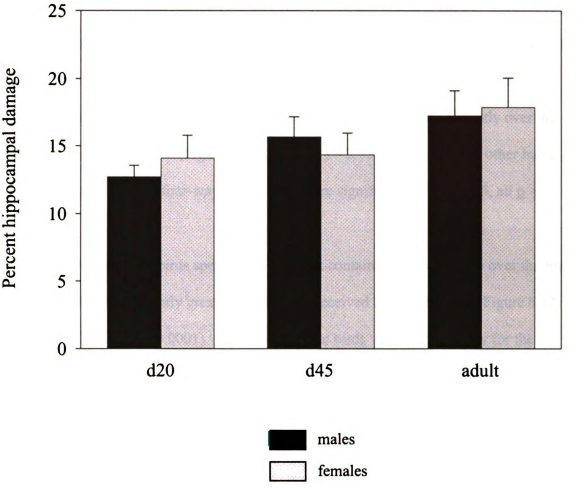


Figure 6.9. Mean (+ SEM) percent hippocampal damage measured in adulthood in male and female zebra finches lesioned at d20, d45 or as adults. Amount of damage to the hippocampus was determined relative to total hippocampus size in each bird. The effect of age of lesion on amount of hippocampal damage measured in adulthood approached significance.

However, latencies to eat from the covered, baited cup during the four probe trials was significantly different between the combined sham and lesion groups (Figure 6.10; $F(2, 141) = 9.83$, $p = 0.003$), and these latencies significantly decreased over the course of the probe trials for both groups ($F(3, 141) = 37.81$, $p < 0.0001$). Further, the mean latencies of males in both the sham and lesion groups decreased more abruptly over the probe trials than all females (Figure 6.11; $F(3, 141) = 2.95$, $p = 0.035$). No other main effects or two-, three-, or four-way interactions were significant (all $F < 1.95$, all $p > 0.077$).

The percent time birds spent in the arm that contained the baited cup over the four probe trials was significantly greater in birds that received the sham lesion (Figure 6.12; $F(1, 141) = 26.02$, $p = <0.0001$). It is expected that birds without a memory for the spatial location of the seed would spend, on average, equal time in both arms ("chance" level). In fact, lesioned birds spent approximately 59% in the goal arm during the first two probe trials, while sham-lesioned birds spent 77 and 84%, respectively (Figure 6.12). Over the probe trials, the amount of time spent in the goal arm increased in both groups ($F(3, 141) = 6.20$, $p = 0.0005$). No other main effects or interactions were uncovered (all $F < 1.41$, all $p > 0.244$).

The most robust effects in this test of spatial memory were the number of mistakes birds made during the probe trials, specifically lifting the flap of or examining a cup that did not contain seed. Overall, lesioned birds made significantly more mistakes than the sham controls ($F(1, 141) = 64.52$, $p < 0.0001$). The number of mistakes depended on the age at which the animal was lesioned ($F(2, 141) = 5.03$, $p = 0.011$), and

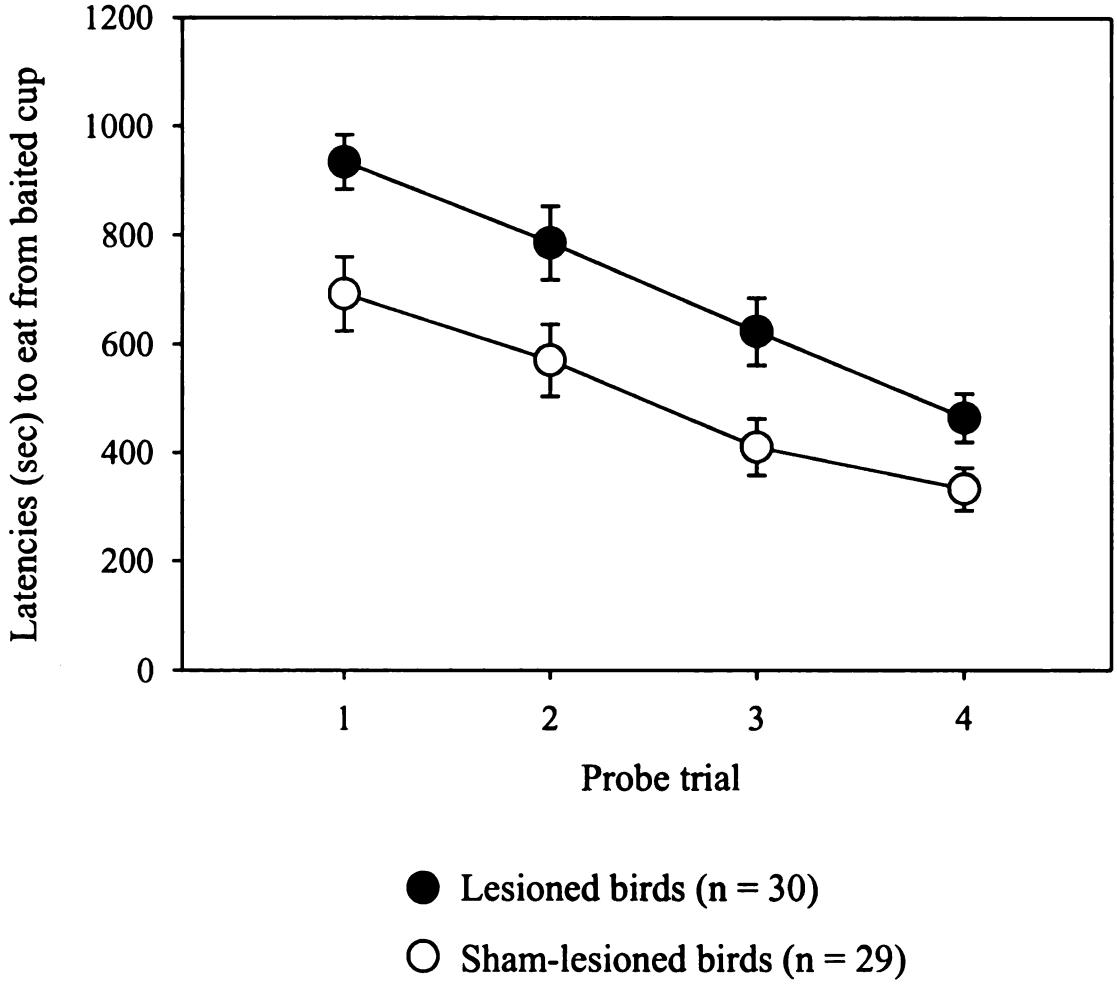


Figure 6.10. Mean (+/- SEM) latencies to eat from the baited cup across the four probe trials for all lesioned and sham-lesioned birds. As a group, lesioned birds differed significantly from sham-lesioned birds, and both groups showed a significant decrease in their latencies across the trials.

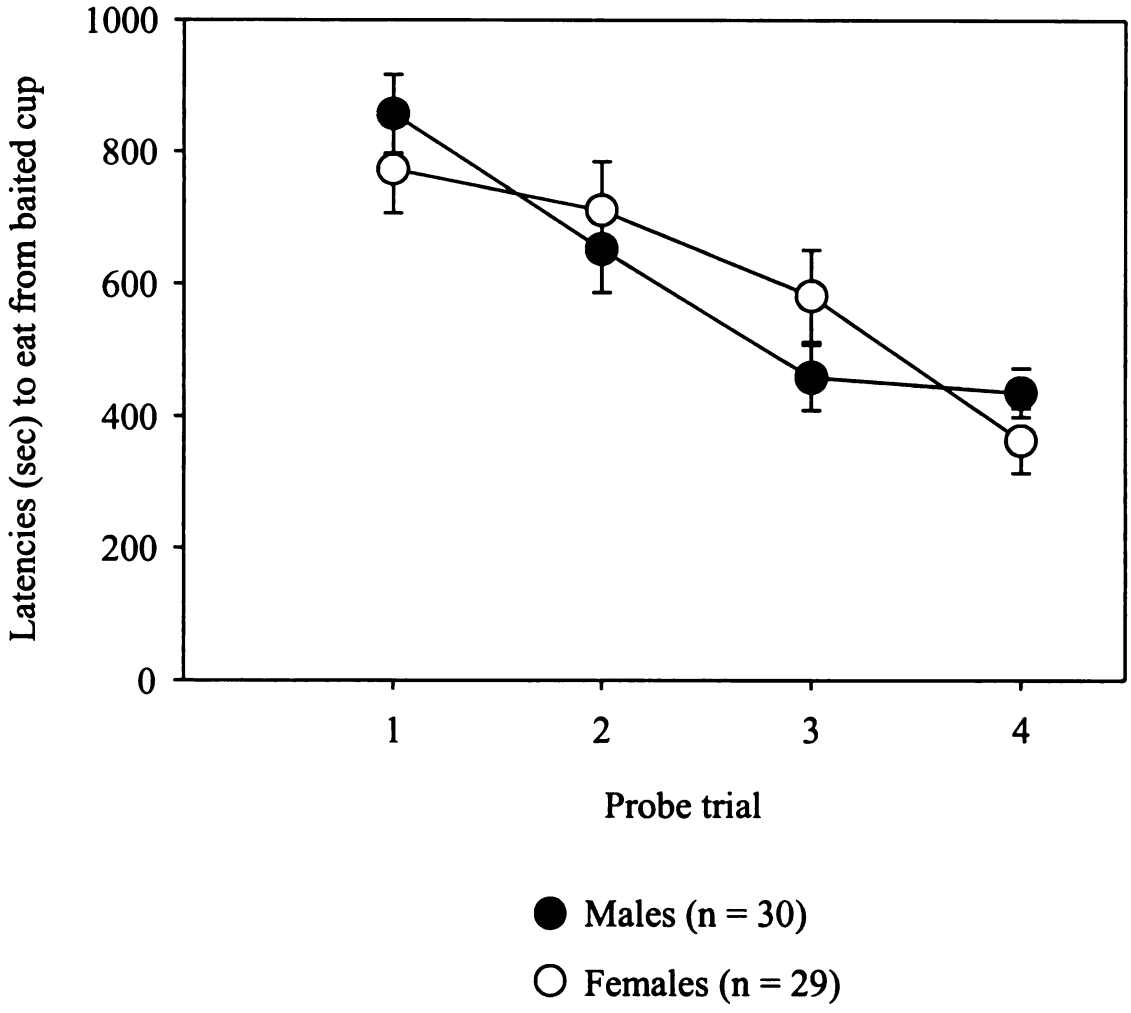


Figure 6.11. Mean (+/- SEM) latencies to eat from the baited cup across the four probe trials for all male and female birds. The latencies of male birds decreased at a significantly faster rate than those of females.

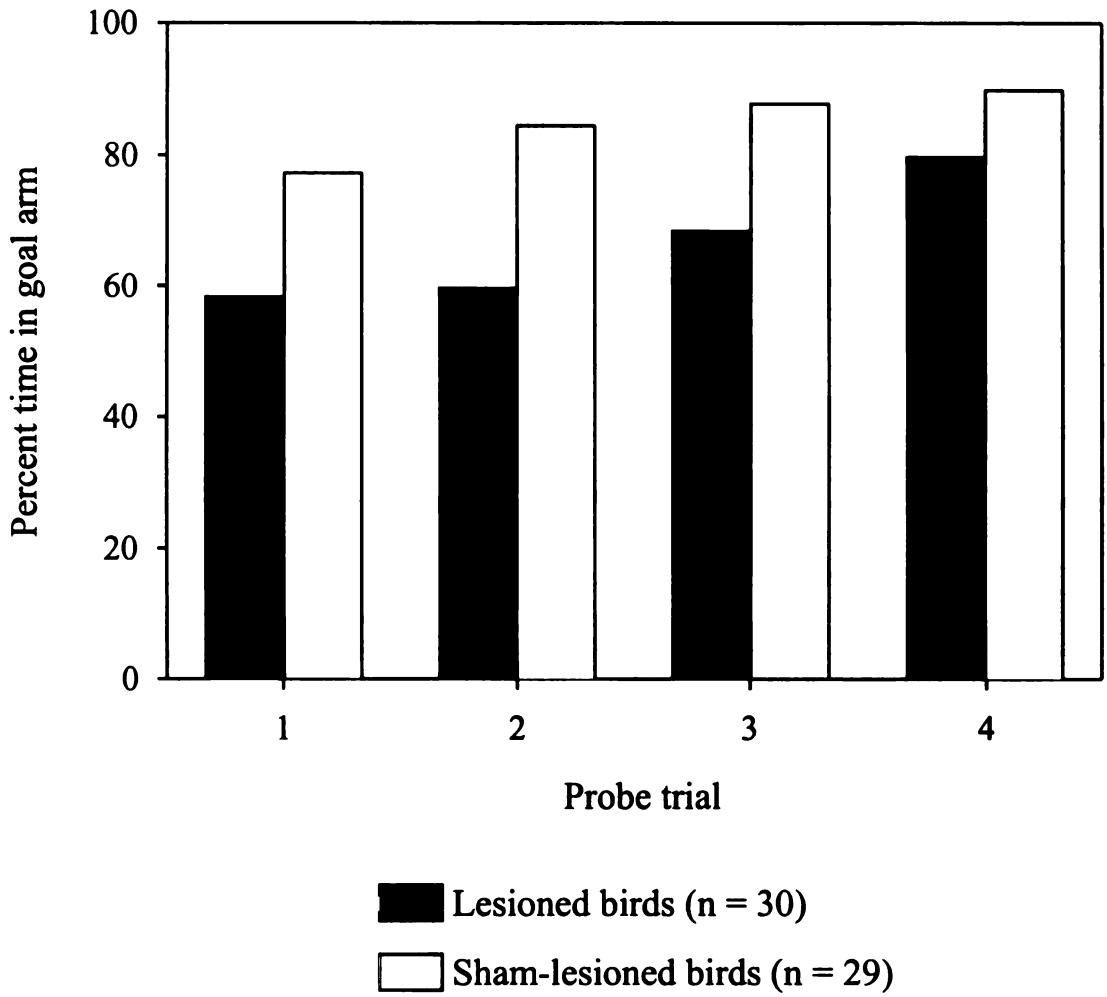


Figure 6.12. Mean percent time in arm with baited cup by all hippocampal-lesioned and control birds across the four probe trials. Over the course of the probe trials, sham-lesioned birds spent significantly more time in the goal arms than lesioned birds.

the number of mistakes made by females tended to be greater than that of males (F (1, 141) = 3.70, p = 0.060). Mistakes decreased over the course of the probe trials for all animals (F (3, 141) = 4.18, p = 0.007) but at different rates for the lesioned and sham-lesioned birds (F (3, 141) = 6.94, p = 0.0002). The four-way interaction of mistakes, age, sex and treatment also approached significance (F (6, 141) = 2.03, p = 0.065), and it was clear that birds made mistakes dependent on these factors (see below). No other interactions were significant (all F < 2.29, all p > 0.112).

Given the main effects and interactions indicated above, mistakes over the course of the probe trials were broken down for further analysis. Examining the first probe trial only (Figure 6.13), lesioned birds made more mistakes than sham-lesioned birds (F (1, 47) = 46.87, p < 0.0001) regardless of age (F (2, 47) = 1.19, p = 0.314), sex (F (1, 47) = 0.18, p = 0.670), or any interaction of those variables (all F < 2.08, all p > 0.137). Examining within the treatment groups, mistakes by the sham-lesioned or lesioned birds were not dependent on age at time of surgery or sex, nor was there an interaction (all F < 2.31, all p > 0.120).

In probe trial two, lesioned birds again made more mistakes than the controls (Figure 6.14; F (1, 47) = 22.96, p < 0.0001) and this was dependent on age at the time of lesion (F (2, 47) = 3.64, p = 0.039). Post-hoc tests revealed that adult birds made significantly more mistakes than d20 birds (Tukey/Kramer; $p < 0.05$); no significant differences were found between d20 and d45 birds or between adults and d45 birds. Also in probe trial two, females made more mistakes than males, although this effect did not reach significance (F (1, 47) = 3.71, p = 0.061). None of the two- or three-

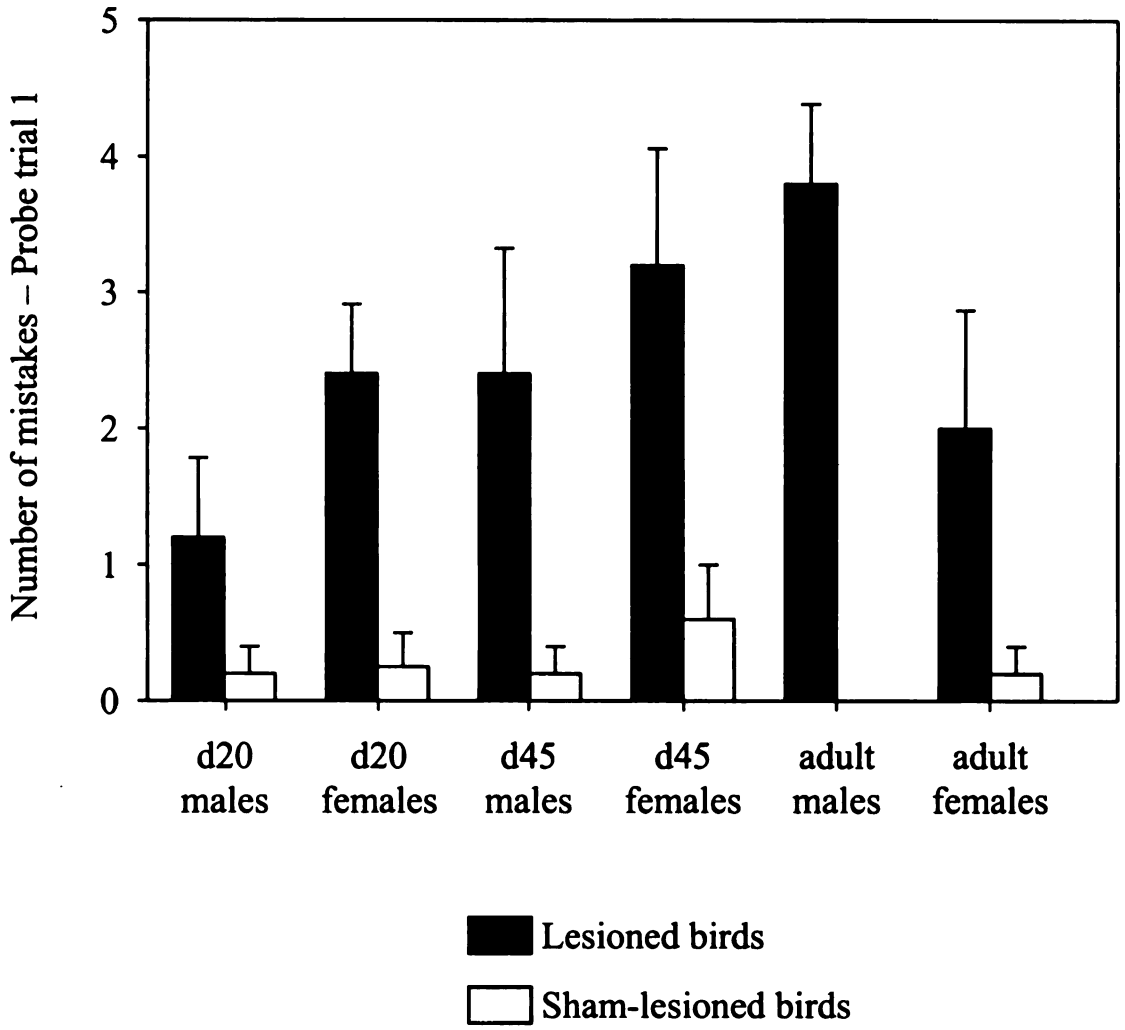


Figure 6.13. Mean (+ SEM) number of incorrect cups examined or flaps of those cups lifted in the first probe trial by adult male and female zebra finches lesioned or sham-lesioned at d20, d45 or in adulthood. Overall, lesioned birds made significantly more mistakes than sham-lesioned ones; there were no significant effects of age or sex or an interaction.

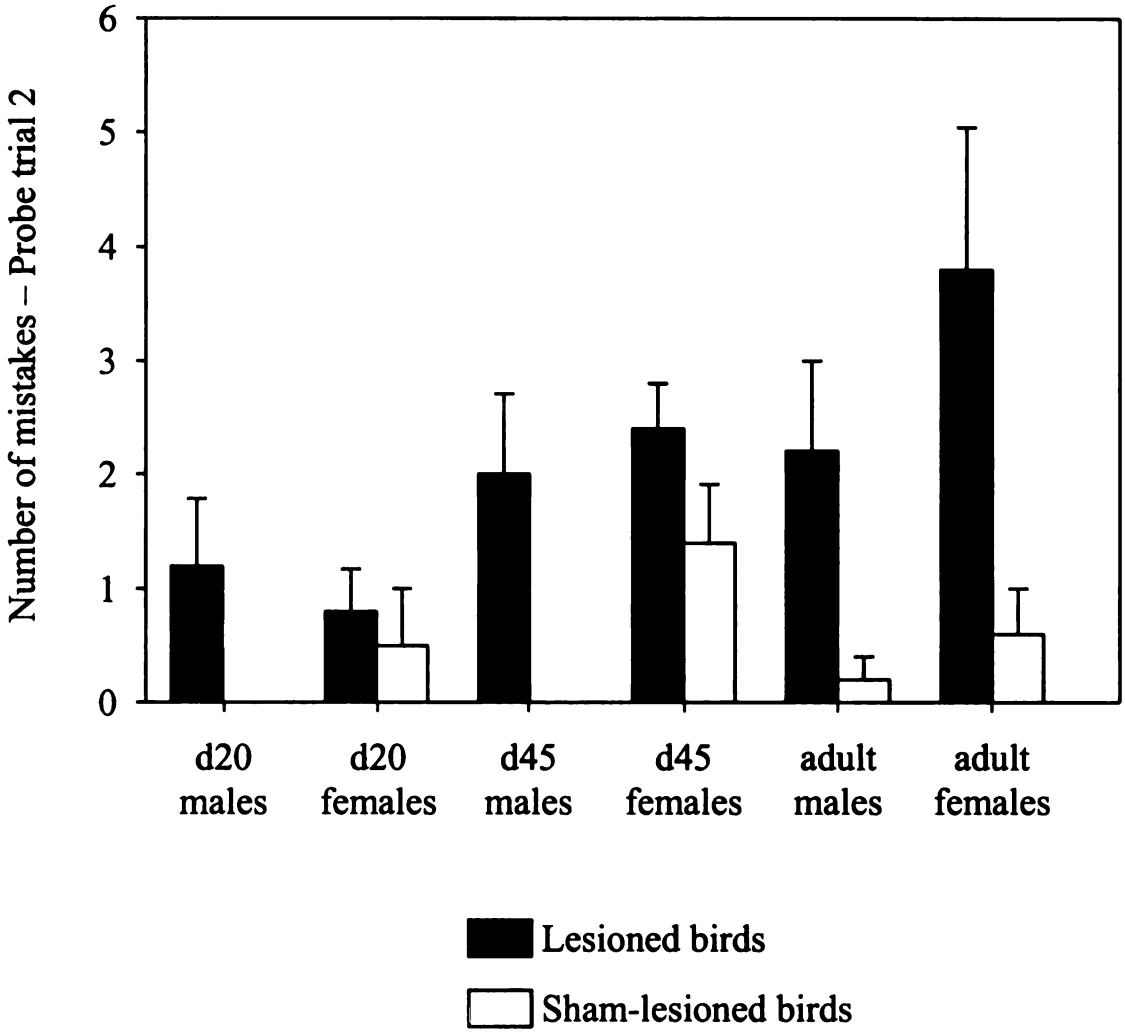


Figure 6.14. Mean (+ SEM) number of incorrect cups examined or flaps of those cups lifted in the second probe trial by adult male and female zebra finches lesioned or sham-lesioned at d20, d45 or in adulthood. Lesioned birds made significantly more mistakes than sham-lesioned ones, and this effect depended on age at time of lesion. Effects of sex approached significance.

way interactions were significant (all $F < 2.52$, all $p > 0.091$). Analysis of mistakes made by sham birds only revealed an effect of sex (F (1, 23) = 7.98, $p = 0.01$): control females made more mistakes than control males. There were no effects of age or an interaction of age and sex (all $F < 1.41$, all $p > 0.264$). Mistakes by lesioned birds in probe trial two were not dependent on sex (F (1, 24) = 0.77, $p = 0.389$) but were affected by age (F (2, 24) = 3.66, $p = 0.041$); there was no significant interaction between the two variables (F (2, 24) = 0.92, $p = 0.414$). Birds lesioned as adults made significantly more mistakes than birds lesioned at d20 (Tukey/Kramer; $p < 0.05$), but no significant differences were found between adult and d45 birds or between the d20 and d45 groups.

In probe trial three, lesioned birds again made significantly more mistakes than sham-lesioned animals (Figure 6.15; F (1, 47) = 47.94, $p < 0.0001$). Birds lesioned at d20 made fewer mistakes than the other groups, and females made more errors than males, although the effects of age and sex only approached significance (F (2, 47) = 2.75, $p = 0.074$ and F (1, 47) = 3.46, $p = 0.069$, respectively). None of the two- or three-way interactions was significant (all $F < 2.24$, all $p > 0.117$). Examining sham birds alone, no significant effects of sex, age or an interaction were uncovered (all $F < 1.40$, all $p > 0.249$). The mistakes by lesioned birds analyzed alone were not significantly influenced by the sex of the animal (F (1, 24) = 2.50, $p = 0.127$), but the effect of age approached significance (F (2, 24) = 2.94, $p = 0.072$). No interaction of sex and age was found (F (2, 24) = 0.99, $p = 0.386$).

More mistakes were made by lesioned birds in probe trial four (Figure 6.16; F (1, 47) = 5.77, $p = 0.021$). No other significant effects or interactions were uncovered when

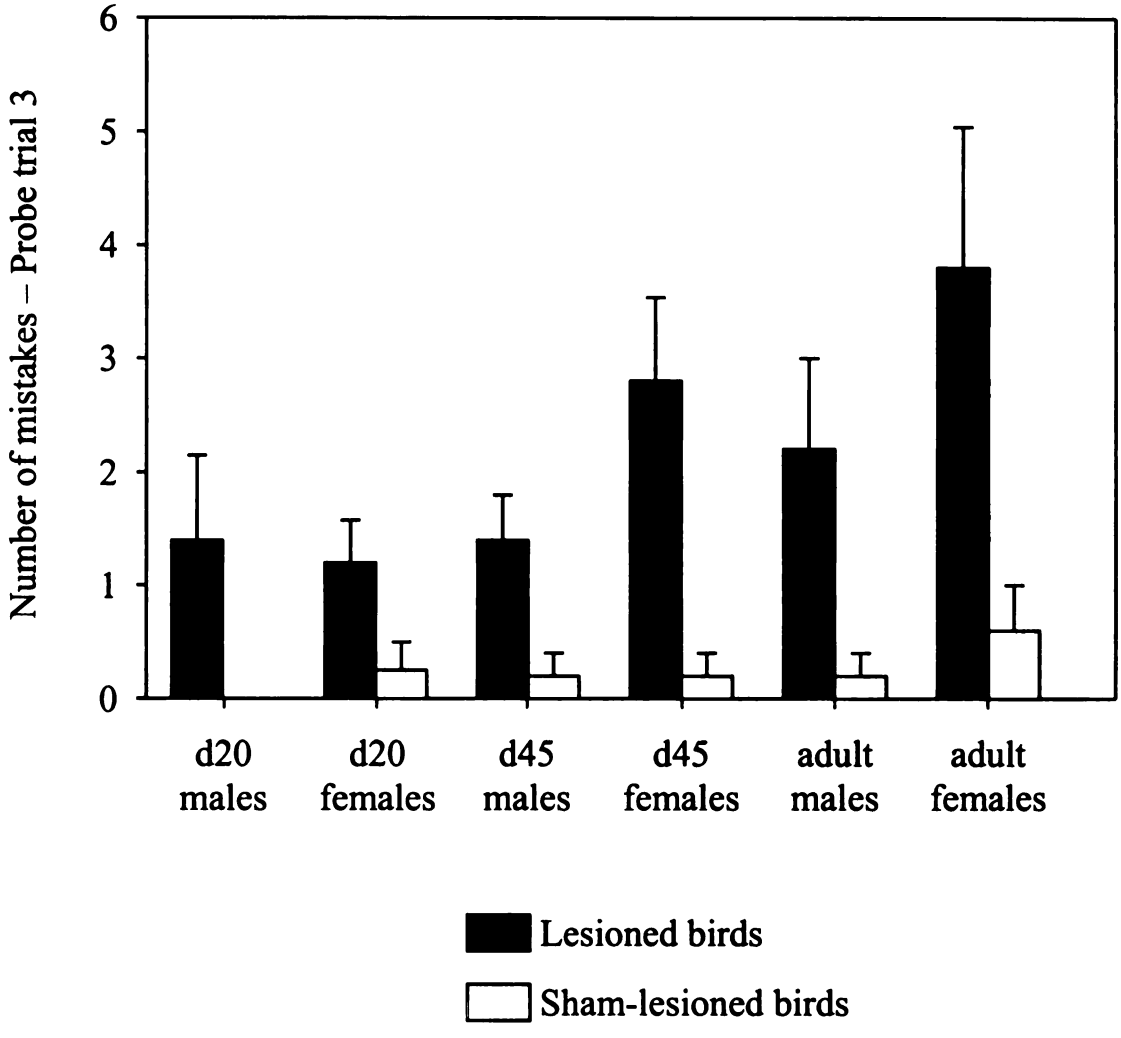


Figure 6.15. Mean (+ SEM) number of incorrect cups examined or flaps of those cups lifted in the third probe trial by adult male and female zebra finches lesioned or sham-lesioned at d20, d45 or in adulthood. Lesioned birds made significantly more mistakes than sham-lesioned ones. Effects of age and sex approached significance.

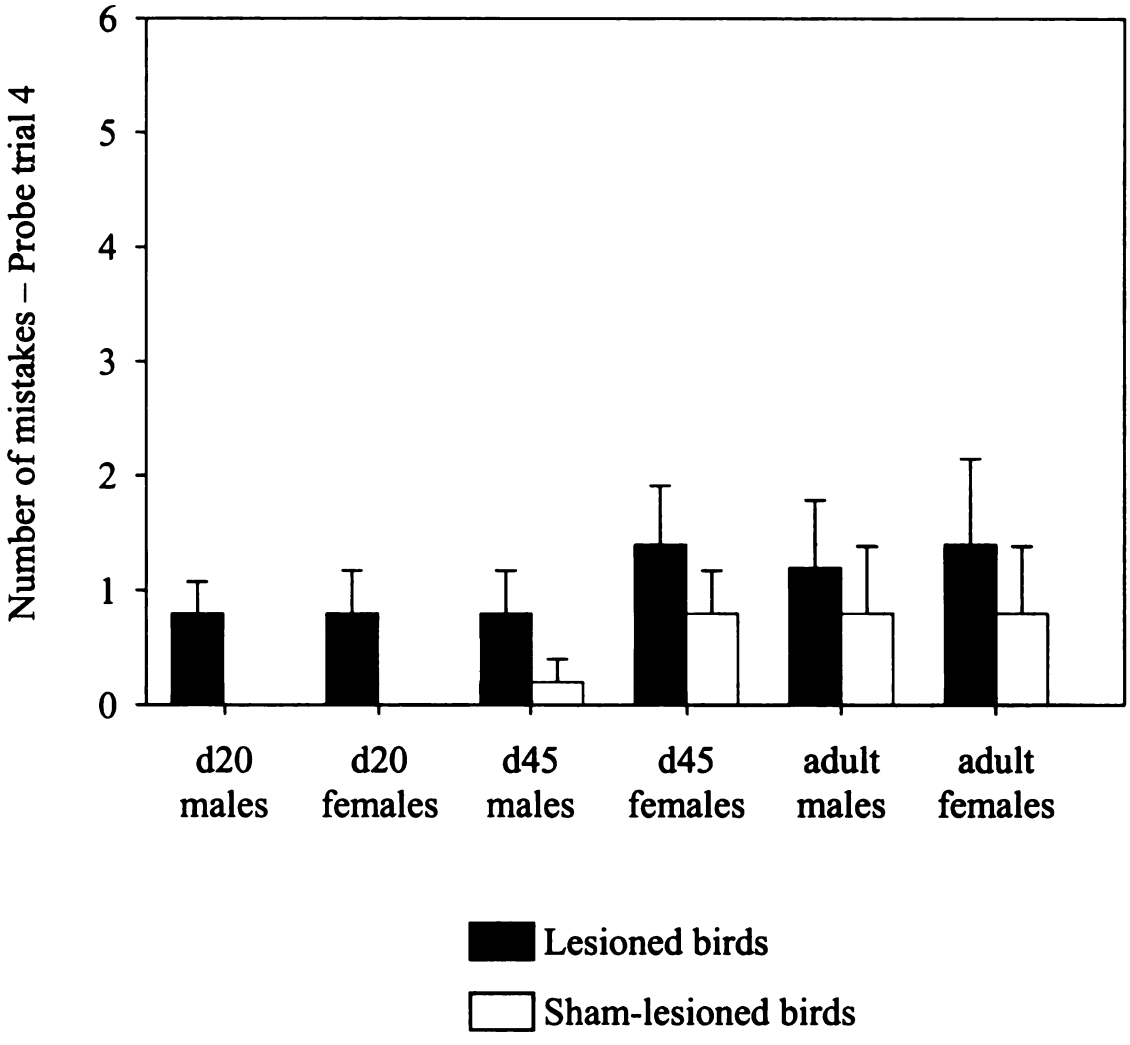


Figure 6.16. Mean (+ SEM) number of incorrect cups examined or flaps of those cups lifted in the fourth probe trial by adult male and female zebra finches lesioned or sham-lesioned at d20, d45 or in adulthood. Lesioned birds made significantly more mistakes than sham-lesioned ones; there were no significant effects of age or sex or an interaction.

all animals were analyzed collectively or when control or lesioned birds were examined alone (all $F < 2.04$, all $p > 0.147$).

Song Preference

Lesions of the hippocampus did not affect the preference for tutor song over the song of a novel male zebra finch (Figure 6.17). No significant main effects were uncovered for age ($F(2, 47) = 0.74$, $p = 0.485$), sex ($F(1, 47) = 0.01$, $p = 0.933$) or treatment group ($F(1, 47) = 0.48$, $p = 0.494$). No significant interactions were found between any of these variables (all $F < 1.06$, all $p > 0.356$). Only one group (females lesioned in adulthood) did not prefer one song over another based on time spent in the part of the chamber broadcasting a particular song (Figure 6.17).

Preference for novel conspecific song over the song of a heterospecific (Figure 6.18) was not affected by lesions of the hippocampus ($F(1, 47) = 0.00$, $p = 0.961$), nor was it significantly influenced by age ($F(2, 47) = 0.41$, $p = 0.669$) or sex ($F(1, 47) = 0.37$, $p = 0.546$). None of the two- or three-way interactions between the variables were significant (all $F < 2.45$, all $p > 0.098$).

Song Production

Ablation of the hippocampus in adulthood did not affect song production in male zebra finches. Percent similarity ($t(8) = 0.33$, $p = 0.747$), mean accuracy ($t(8) = 0.40$, $p = 0.699$), percent sequential match ($t(8) = 1.07$, $p = 0.317$) and overall similarity scores ($t(8) = 0.45$, $p = 0.665$) did not significantly differ between the groups based on the comparisons of phrases from songs recorded pre- and post-sham or lesion (Table 6.3).

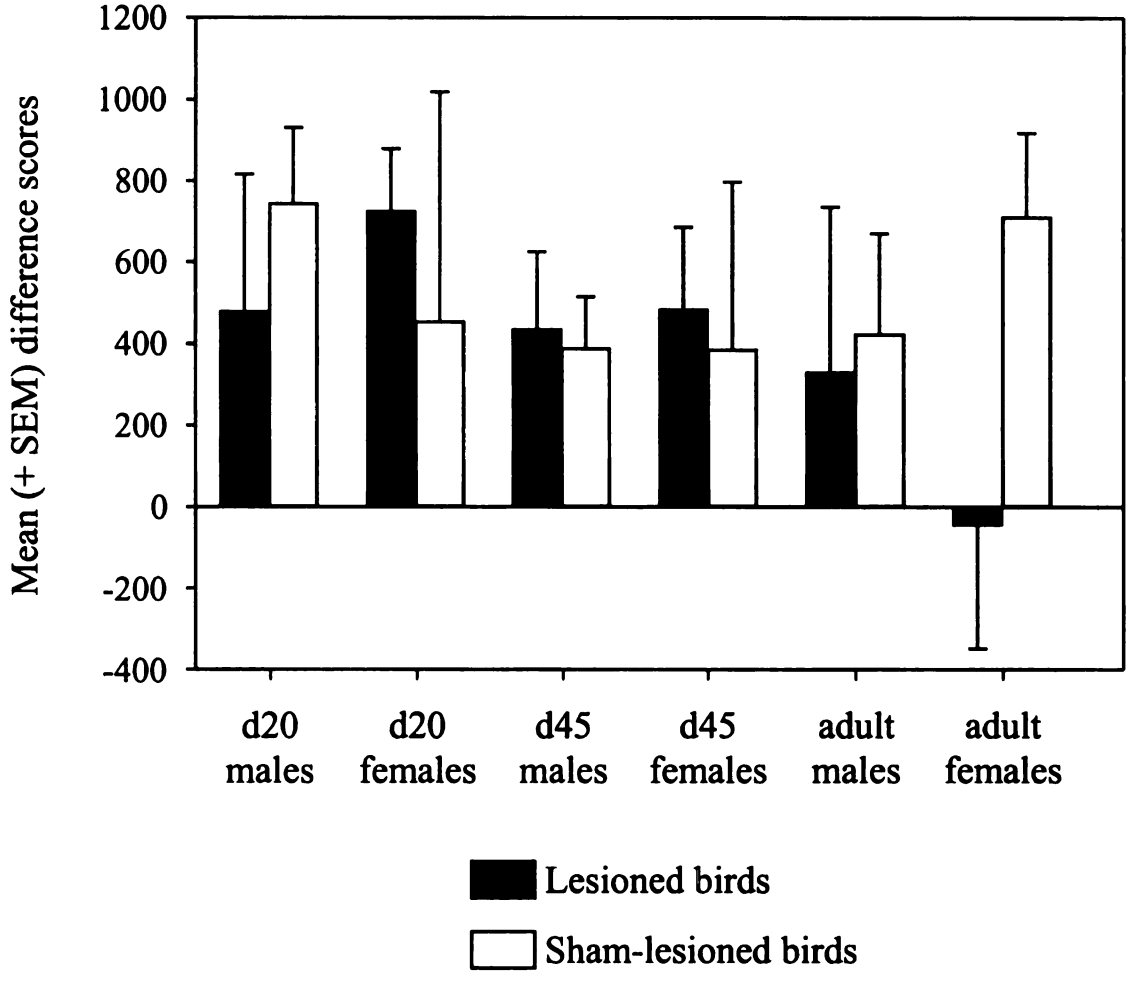


Figure 6.17. Mean (+/- SEM) difference scores (amount of time (sec) spent in side of chamber playing father's song minus amount of time spent in side of novel male conspecific song) of male and female zebra finches hippocampal-lesioned or sham-lesioned at 20 days post-hatch (d20), d45 or adulthood. A positive score indicates a preference for the father's song, and a negative score a preference for the song of a novel male conspecific. No significant main effects or interactions were uncovered.

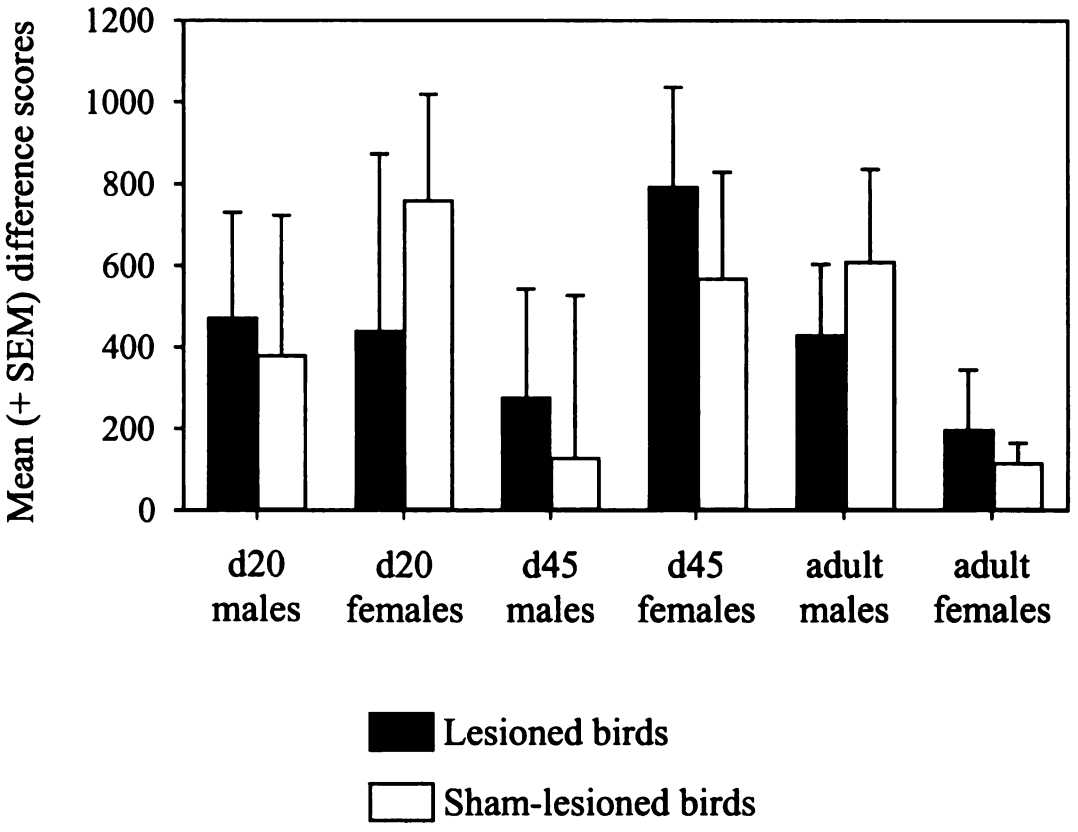


Figure 6.18. Mean (+ SEM) difference scores (amount of time (sec) spent in side of chamber playing conspecific song minus amount of time spent in side of heterospecific song) of male and female zebra finches hippocampal-lesioned or sham-lesioned at 20 days post-hatch (d20), d45 or adulthood. No significant main effects or interactions were uncovered.

		Means (+/- SEM)			
Group	Treatment	Similarity Score	Percent Similarity	Mean Accuracy	Sequential Match
d20	lesion	64.3 (5.9)	92.6 (1.5)	77.4 (0.6)	80.2 (17.7)
d20	sham	55.6 (3.5)	84.4 (3.8)	71.9 (2.5)	83.7 (6.8)
d45	lesion	65.9 (2.3)	89.8 (2.7)	74.4 (2.1)	95.0 (5.0)
d45	sham	64.6 (5.0)	84.4 (2.4)	76.5 (2.1)	90.3 (6.0)
adult	lesion	86.3 (3.2)	96.8 (0.7)	92.8 (2.8)	96.9 (0.5)
adult	sham	88.7 (4.25)	96.0 (2.3)	94.1 (1.5)	98.1 (1.1)

Table 6.3. Similarity measurements of song phrases from adult male zebra finches lesioned or sham-lesioned at d20, d45 or in adulthood. For d20 and d45 birds, similarity measurements were obtained by comparing one phrase from the songs of each of these males with one from their father. In birds lesioned or sham-lesioned as adults, phrases from individual males' songs both before and after lesion (or sham lesion) were compared. None of the similarity measurements from the lesioned birds differed significantly from those of the sham controls.

No additional notes or changes in note order were detected in any of the adult-lesioned birds (Figure 6.19).

Analyzed together, the overall similarity scores of birds lesioned at d20 or d45 did not significantly differ (main effect of age: $\underline{F}$ (1, 16) = 1.47, $\underline{p}$ = 0.243; treatment: $\underline{F}$ (1, 16) = 1.30, $\underline{p}$ = 0.271; Table 4). Percent similarity with a tutor's song phrase was not influenced by age of lesion ($\underline{F}$ (1, 16) = 0.27, $\underline{p}$ = 0.613) but was, interestingly, higher in the lesioned animals compared to the sham controls ($\underline{F}$ (1, 16) = 6.29, $\underline{p}$ = 0.023). The mean accuracy and sequential matches between tutor and pupil's song phrases was not significantly affected by the hippocampal lesion ($\underline{F}$ (1, 16) = 0.79, $\underline{p}$ = 0.387 and $\underline{F}$ (1, 16) = 0.004, $\underline{p}$ = 0.952) or age at time of lesion ($\underline{F}$ (1, 16) = 0.17, $\underline{p}$ = 0.689 and $\underline{F}$ (1, 16) = 1.10, $\underline{p}$ = 0.310, respectively). No significant interaction between age and group was uncovered through analysis of any of the song similarity measures (all $\underline{F} < 3.73$, all $\underline{p} > 0.071$).

Regression Analyses

To confirm whether the extent of a lesion correlated with behavior on a particular task, simple regressions were performed within each age group for each dependent variable in the experiment: initial latency to eat from the baited cup during the first acquisition trial, number of trials to reach criterion, latencies to eat from the baited cup, percent time in the goal arm, and mistakes during the four probe trials, difference scores in the preference tests (time in side of chamber broadcasting father's song minus side playing novel male conspecific song and novel conspecific song minus heterospecific

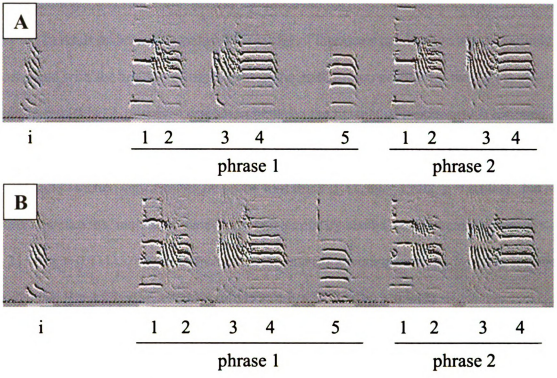


Figure 6.19. Spectral derivatives of samples of the songs of a male zebra finch before (A) and after (B) a bilateral lesion of the hippocampus. Note that the types of notes used and the order of those notes are identical before versus after the lesion.

song), and song similarity measurements (overall similarity score, percent similarity, mean accuracy and sequential match) for males only.

In adult males and females, the amount of hippocampal damage was negatively correlated with the amount of time spent in the goal arm on probe trial two (F (1, 9) = 5.64, p = 0.049). A positive correlation between the percent of hippocampus lesioned and number of mistakes approached significance on probe trial two (F (1, 9) = 5.05, p = 0.055) and reached significance on probe trial three (F (1, 9) = 13.02, p = 0.007). No other correlations, including males only and similarity scores, were uncovered (all F < 3.21, all p > 0.111). No correlations on any measure were uncovered for birds lesioned at d20 (all F < 4.05, all p > 0.079) or d45 (all F < 8.37, all p > 0.063).

DISCUSSION

Lesions of the dorsolateral subdivision of the hippocampus, where I detected immediate early gene activity in response to conspecific songs (Bailey et al., 2002; Bailey and Wade, 2003; Bailey and Wade, 2005), had no effect on (1) the ability of birds lesioned during development to learn song in order to normally respond to and produce it in adulthood or (2) song stability and responses to song in adulthood, following lesions of the region in developed animals. These results suggest that immediate early gene activity in the hippocampus does not confer a role for the region in song learning *per se*, but they do not exclude the possibility that the structure consolidates memories relating to song or modulates the responses of other regions based on the salience of particular vocal signals or environments in which they are heard or produced. Although not the primary focus of this study, the spatial memory task developed is a valid and reliable tool with which to

test the ability of normal male and female zebra finches to remember the location of a particular stimulus in a fairly complex environment and to measure the attenuation in that behavior that results from specific neural damage.

Ablations of different regions within the song circuit in zebra finches have resulted in specific effects on song behavior. For example, lesions of Area X and lMAN during the sensitive period for song learning prevent normal song development, specifically by affecting the circuitry of the motor nucleus RA in the case of lMAN lesions(Kittelberger & Mooney, 1999), while similar lesions in adult birds do not affect song (Bottjer et al., 1984; Scharff & Nottebohm, 1991). Destruction in development or adulthood of cells within HVC and RA disrupts song output (Scharff & Nottebohm, 1991; Simpson & Vicario, 1990). The present results clearly indicate that the hippocampus does not influence song learning or production like these "traditional" song control nuclei. Based on similarity scores with tutor phrases, lesions of the hippocampus at d20 and d45 did not disrupt formation of a song template or the later sensorimotor integration required for song stabilization. In adult males, song remained stable following the hippocampus lesion, again suggesting no involvement of the region in song maintenance or expression of the song template. In all birds, normal song preferences for tutor song over that of a novel male conspecific and conspecific over heterospecific song were maintained, unlike, for example, lesions of CMM in adult female zebra finches that result in the display of similar responses to both heterospecific and conspecific song (MacDougall-Shackleton et al., 1998).

The question remains as to why, given that neurons in the hippocampus respond selectively to conspecific songs, do lesions of the structure not impact normal song

preferences? Other than the possibility of encoding information about the environment in which relevant auditory signals are heard (discussed in further detail below), the hippocampus may modulate the activity of other regions to apportion proper responses to specific song stimuli. Evidence for this potential function of the hippocampus comes from male zebra finches that underwent their first courtship of a female: FOS immunoreactive neurons were observed throughout the hippocampus (Sadananda & Bischof, 2002), expression that was higher than that seen in males from an aviary with social and sexual contact with conspecifics or males chased around a cage by a human experimenter. Again, this activity within the structure broadens the role of the hippocampus over and above spatial memory processing. Immediate early gene activity in the hippocampus following acquisition in a spatial memory paradigm (see Herdegen & Leah, 1998) may not necessarily infer the sole function of the region during the task. Hippocampal c-FOS mutant mice exhibit normal spatial learning compared to control mice in the Morris water maze task, suggesting no involvement of the immediate early gene in this type of memory consolidation. Therefore, activity within the region may be modulatory in nature, affecting other brain regions to gauge the relevance of particular situations and stimuli, and linking them appropriately with some sort of behavioral response.

The most robust findings of the present study are the differences in spatial memory ability between control birds and hippocampal-lesioned animals that mirrors effects obtained in this and other avian species and provides further evidence for a functional homology with the mammalian hippocampus. Food-deprived zebra finches with lesions to the hippocampus are deficient in their ability to locate a previously found

food source (Patel et al., 1997a; Watanabe & Bischof, 2004). Also, spatial memory in pigeons (Bingman, Bagnoli, Ioalé, & Casini, 1984) and black-capped chickadees (Sherry & Vaccarino, 1989) is disrupted by lesions to the hippocampus. In the present study, lesioned birds displayed significantly higher latencies than the controls to eat from the baited cups during the probe trials, spent significantly less time in the arm that contained the seed, and made more mistakes at locating the food than the sham-lesioned animals. Interestingly, the performance of lesioned animals increased over the course of the probe trials. A gradual decrease in latencies to eat from the baited cup, an increase in the time spent in the arm that contained the cup, and decreases in mistakes over the probe trials all indicate, perhaps, that alternate strategies were used to determine the location of the food source and that learning occurred outside of the realm of spatial processing as in a prior study (Watanabe & Bischof, 2004), or that what remains of the hippocampus could function better with time. The mechanisms through which this compensation may occur have not been studied in avian species. It is possible, however, that the 10 min interval between probe trials was short enough to permit birds in both groups to retain the location of the baited cup in short-term memory. There is no doubt, however, that lesioned birds were initially deficient in spatial long-term memory based on their performance in the first probe trial, which followed the acquisition trials by 1 hr and is thus outside the domain of short-term memory.

Two other findings from the present results on spatial memory ability in zebra finches also warrant further attention. First, the performance of birds lesioned at d20 was better than adults on a few measures (see Results). Also, although not statistically different, the percent lesion to the DLHP was lower in birds lesioned at d20 than those

lesioned in adulthood. To my knowledge, neurogenesis in the zebra finch hippocampus has not been reported, although environmental change induces neurogenesis in other brain regions, such as HVC and Area X (Lipkind, Nottebohm, Rado, & Barnea, 2002). Given the occasional increased performance of these birds, their smaller lesion volume, that neurogenesis within the avian brain (Nottebohm, 2002a; Patel, Clayton, & Krebs, 1997b) is well documented, and that the hippocampus in mammals is a primary site for functional neurogenesis (van Praag, Schnider, Christie, Toni, Palmer, & Gage, 2002), this is a possibility. It is also unknown whether the lesion occurred before a period of increased development of the hippocampus, such that an increase in cell birth within the region following the lesion may have compensated slightly for the loss of neurons.

Also interesting is the occasional sex difference in ability to remember the location of the baited cup. Females made more errors than males in some of the probe trials, and their latencies to eat from the baited cup declined at a slower rate than males over the four probe trials. Males of many species show consistent advantages in a variety of spatial tasks (see Jonasson, 2005). Zebra finches do not migrate, are not territorial, and both sexes forage for food and take care of the young (Zann, 1996), which suggests equal spatial memory demands between the sexes, yet several instances of male-biased performance are seen. Although this difference is not robust throughout the probe trials, due possibly to the number of subjects in each group, additional questions remain as to whether the morphology, neurochemistry or electrophysiological activity of the hippocampus differs between the sexes in the zebra finch, or whether differences in non-spatial learning tasks exist.

This experiment serves as an important first step in determining the involvement of the hippocampus in song-related behavior. Although the region does not appear to be important for song learning directly, the song-specific responses of the region (Chapters 3 and 4), its afferent and efferent connections (detailed in Chapter 5), and its role in spatial memory (current Chapter) are suggestive of, at the very least, a modulatory role in vocal communication in zebra finches.

CHAPTER SEVEN

Zebra finches are an ideal model system for the examination of multiple memory systems, in particular for exploring how they develop and work in concert to control discrete, readily observable behaviors. The results presented in the preceding chapters begin to illustrate several points regarding particular aspects of song learning in female zebra finches, neuronal responses to song during development, and the potential role of the hippocampus in song-related behavior in zebra finches. These points are summarized and expanded on in the sections below.

Learned song behavior may not be restricted to a sensitive period in female zebra finches

Male zebra finches learn song during a distinct period in development in order to later produce it in adulthood to attract a female (Immelmann, 1969). Females do not sing, but are selective in their responses to particular song stimuli, suggesting that they, too, may learn song as males do (see Riebel, 2003). As detailed in Chapter 2, females isolated from song but not biparental care during development showed normal responses to the playback of conspecific songs in adulthood but their responding to the environment in which conspecific (but not heterospecific) songs were heard 24 hours earlier was attenuated. However, as the number of song exposures increased, the responses of song isolated females increased to the levels of birds raised normally. These results suggest that (1) female zebra finches may differentially form representations of environments in which relevant songs are heard depending on prior exposure to those songs and (2) that experience with song in adulthood can overcome deficits in responding to song due to developmental isolation from it, similar to the ability of male zebra finches to learn song

outside of the traditionally-defined sensitive period (Chapter 2; Clayton, 1987; Eales, 1985, 1987a; Jones et al., 1996).

The interesting points that result from this behavioral study are the apparent ability of the female zebra finch brain to remain plastic into adulthood and the specific neural mechanisms that may be involved in the formation of memory for conspecific songs or, perhaps, the ability of a bird to associate salient vocal communication with a particular environment. The brains of males and females show distinct neuronal responses to song that can be measured by the activity of immediate early genes (see Chapters 1, 3 and 4). Through this type of analysis, I have shown that the hippocampus responds to conspecific song stimuli more so than other types of auditory signals, suggesting a role in the consolidation or modulation of behaviors necessary for the generation of proper responses to song. If, as hypothesized, conspecific song-induced activation in the hippocampus is important for the consolidation of a "contextual song memory (Chapter 1)," then significantly less immediate early gene expression would be expected in the hippocampus of a female isolated during development (as in this study) and exposed to playback of normal conspecific song. Although females in the experimental group were not exposed to normal vocalizations from their fathers during development, these males still produced sounds associated with respiration. It is possible that song isolated females formed memories of their fathers' non-song vocalizations during development. If this is correct, then song isolated females should at least initially prefer this auditory stimulus in a test of song preference in adulthood. Additional insight into the function of cells in the hippocampus could also be obtained: immediate early gene induction in the hippocampus of song isolated females following playback of

vocalizations from axotomized fathers should be as robust as the responses of neurons in the hippocampus of birds raised normally and presented with normal zebra finch song.

Immediate early gene responses of neurons in auditory regions of male and female zebra finches change over development as song learning progresses

The bulk of the published data regarding this immediate early gene expression in neurons in response to song have used adult birds. In adult male and female zebra finches, song playback induces immediate early gene expression in NCM (Bailey et al., 2002; Mello et al., 1992), CMM (Bailey et al., 2002; Bolhuis et al., 2000) and the hippocampus (Bailey et al., 2002; Bolhuis et al., 2000; Kimpo & Doupe, 1997; Kruse et al., 2004). The data I collected, along with that from others, have established that neuronal responses to song at two periods in development, when song templates begin to be acquired (d30) and when the sensorimotor integration period in males begins (d45), are differential between males and females (at d30) and over time equalize (between d30 and d45). At d30, neuronal responses to song in female zebra finches are indicated via FOS expressing neurons, and those in males via ZENK expressing cells. This differential activation indicates the value of investigating the responses of both FOS and ZENK, and future studies that examine auditory responses in songbirds would benefit from employing antibodies to the protein products of both genes, especially studies involving both males and females. Also in d30 females, the hippocampus is the only region in which conspecific song significantly increases immediate early gene activity compared to other auditory stimuli (Chapter 3). At d45, responses in the two sexes equalize, and the NCM is the only structure in which significant effects of auditory stimuli are uncovered

(Chapter 4). This suggests that the hippocampus may be important for song learning in females during what could be their sensitive period (between d25-35; see Chapter Two), and by d45, the NCM is the primary region of the brain in both sexes that responds to conspecific song stimuli. Although many nuclei in the zebra finch brain are sexually dimorphic (Nottebohm & Arnold, 1976), in adulthood the gross morphological structure as well as the function of the auditory regions NCM and CMM appear equivalent between the sexes. Why cells of these regions initially respond to song via divergent neural mechanisms and how and why these cellular responses equalize over development remain unknown.

Potential functional significance of immediate early gene activity following song presentations

The great interest that has surrounded the examination of the expression of immediate early genes in the brain has been their potential involvement in synaptic plasticity that could result in long-term memory formation. Indeed, this hypothesis has been reproduced in countless studies across diverse species in which immediate early gene activation correlates with acquisition of some particular behavior, suggesting involvement in learning (see Tischmeyer & Grimm, 1999).

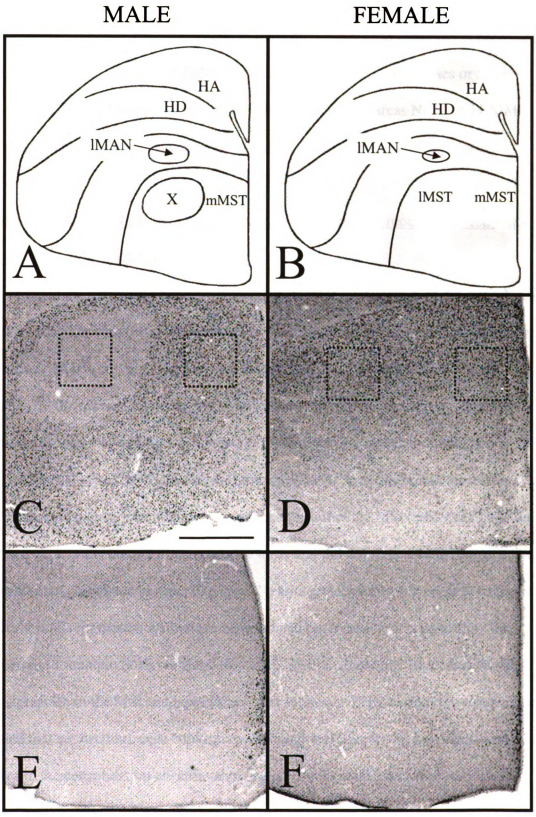
Immediate early gene induction triggers a cascade of post-transcriptional activity that affects signal transduction, neuronal excitability, synaptic function or cytoskeletal structure (Clayton, 2000; Mello, 2002). While induction of these immediate early genes is only one of several regulatory events that take place following neuronal activation, it is tempting to speculate in zebra finches what this activation may modify. For example,

additional data I have collected point to one such mechanism that may promote long-term memory formation related to song. Area X of the medial striatum (MSt) is present in males and integral to song learning (see Chapter 1) but is not identifiable via a Nissl stain in females. In d30 and d45 birds, ZENK is not induced in Area X in males following presentations of conspecific song but is activated in the surrounding MSt. ZENK expression is homogenous throughout MSt in females, including the region that corresponds to Area X (Figure 7.1). Neurochemical data from other studies show a similar pattern of expression. Neurons immunoreactive for an antibody to the NMDA receptor subunit NR1 are not detected within Area X in adult male zebra finches but are found in the surrounding striatum; interestingly, these NR1-immunoreactive cells are found throughout the striatum in females (Saldanha et al., 2004). These patterns of expression suggest a potential interaction between ZENK and NMDA receptors in song development. For example, it is possible that ZENK induced by hearing conspecific song influences the expression of NR1 in the MSt excluding Area X in males but throughout the region in females. ZENK is integral in the modulation of neuronal excitability (reviewed in Knapska & Kaczmarek, 2004) and expression of zif-268 (of which ZENK is an avian homolog) in rodents appears to play a role in maintaining plastic changes associated with long-term potentiation (LTP) (Knapska & Kaczmarek, 2004). Glutamatergic receptors are important in LTP as well (Nordeen & Nordeen, 2004), and activation of them during song template acquisition is vital to song learning and perhaps even the opening of the sensitive period for it (Aamodt, Nordeen, & Nordeen, 1996; Basham, Nordeen, & Nordeen, 1996; Heinrich, Singh, Sohrabji, Nordeen, & Nordeen, 2002; Nordeen & Nordeen, 2004; Scott, Singh, Nordeen, & Nordeen, 2004; Singh,

Basham, Nordeen, & Nordeen, 2000). The next step in understanding the function of immediate early genes, specifically how they relate to song learning and perception, is an analysis of what they are co-expressed with at various developmental stages following presentations of conspecific song. To start, analysis of the co-expression of ZENK and NMDA receptors under conditions of song exposure can lead to more of an understanding of their roles in influencing song learning and behavior.

In addition to work needed to further evaluate the cascade of cellular events triggered or modified by immediate early gene induction, future studies should correlate immediate early gene expression with a bird's observable preference for a stimulus. In my work with adult females (Bailey et al., 2002) as well as d30 (Bailey and Wade, 2003) and d45 (Bailey and Wade, 2005) males and females, auditory stimuli were presented in the dark to minimize immediate early gene induction associated with visual stimuli and to replicate the conditions of prior work (Bolhuis et al., 2000) in zebra finches. Because of this, only auditory recordings were made while birds underwent the acclimation, song exposure and post-song exposure periods. Hardly any vocalizations were made by birds, and although movement was occasionally heard, it was of course impossible to know in detail how the birds responded to the song stimuli. Recent work in female white-crowned sparrows quantified both the ZENK response and behavioral responses to song of hatch or local dialect (which is preferred) versus foreign dialect. Similar to results obtained with zebra finches, ZENK induction in the NCM and CMM was greater in the group exposed to the preferred song of local dialect (Maney et al., 2003). Additionally, ZENK immunoreactivity in NCM was correlated with a non-vocal copulation solicitation display, "wing quivers," and induction in CMM with a vocal copulation solicitation

Figure 7.1. Drawings of coronal sections through the zebra finch brain detailing the structures visible via Nissl stain (panels A and B). In males (A), Area X is evident as a large nucleus distinct from the surrounding medial striatum (MSt). Area X is not detectable in the female MSt (B). The remaining panels are photomicrographs of coronal sections through the MSt of juvenile (d30) zebra finches detailing ZENK and FOS immunoreactivity following conspecific song presentations. Note the almost complete absence of ZENK immunoreactivity in Area X in males (panel C, dotted box on left) compared to the relatively homogeneous immunoreactivity throughout the MSt in females (dotted boxes in panel D). Little to no FOS immunoreactive neurons were detected in both the male (E) or female (F) MSt. The midline is at the right in each section. HA = hyperpallium apicale, HD = hyperpallium densocellulare, lMAN = lateral portion of the magnocellular nucleus of the anterior nidopallium, X = Area X of the medial striatum (MSt), mMST = medial portion of the medial striatum, lMSt = lateral portion of the medial striatum. Scale bar = 1.0 mm.


MALE
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display, "trills." The behavioral correlate to immediate early gene induction is necessary to further deduce the function of ZENK and FOS, for example, in responses or future responses to song, and how their activation within the auditory areas NCM and CMM influences the motor regions necessary for production of observable preference.

The hippocampus and NCM in zebra finches may be involved in modulating responses to the environmental or social contexts of song

Immediate early gene expression is observed in several regions of the avian brain following context-dependent song production, perception or spatial memory performance. For example, in homing pigeons that were transported to a familiar training site and released, ZENK immunoreactivity was four times greater in the lateral portion of the hippocampus than that in birds transported to the familiar site and that did not home (Shimizu, Bowers, Budzynski, Kahn, & Bingman, 2004). In addition, two times more ZENK expressing cells were found in the medial portion of the MSt (mMSt) in birds that navigated back to the home loft compared to birds that did not. The authors suggest that the mMSt may participate in route like spatial learning that operates in parallel with more maplike spatial representations that are consolidated by, stored in or mediated by the hippocampal formation (also see Gagliardo et al., 1999). Given that immediate early gene expression in the MSt and hippocampus are highest in birds exposed to conspecific song and that connections exist between the two regions (Chapter 5), they may work together in the consolidation or retrieval of spatial or relational memories, such as the "contextual song memory" hypothesized above.

An additional experiment I designed further examines the contextual activation within auditory regions by determining whether the activity-regulated cytoskeletal-associated protein (ARC), an immediate early gene modulated by synaptic activity (Guzowski, Lyford, Stevenson, Houston, McGaugh, Worley, & Barnes, 2000; Guzowski, Setlow, Wagner, & McGaugh, 2001; Lyford, Yamagata, Kaufmann, Barnes, Sanders, Copeland, Gilbert, Jenkins, Lanahan, & Worley, 1995), is differentially expressed in NCM following exposure to particular auditory and/or non-auditory stimuli. In this experiment, female zebra finches were moved from group housing to individual cages in a 'home' environment, and after an acclimation period of one day were presented with song or no auditory stimulus in either that room or a new one. Some of the birds were euthanized, and others were returned to their home facility until the following day, at which time they were either re-exposed to the same or a novel combination of visual and auditory stimuli. In both cases, the density of somatic and dendritic ARC immunoreactivity was determined (Figure 7.2). Increased ARC immunoreactivity in NCM was observed only in females exposed to song following a change in environment; this effect existed under both single and double exposure conditions (Bailey, Beck, Svec, & Wade, 2005). Surprisingly, levels of ARC in each subdivision of the hippocampus (dorsolateral, dorsomedial and ventral) were not affected by song exposure or change in environment. These data suggest a role for ARC in the ability of NCM neurons to associate environmental and conspecific song stimuli, and parallel data from others (Kruse et al., 2004) that suggest non-auditory stimuli (such as the change of environment in the present study) influence levels of ZENK expression within the NCM. Work is ongoing to determine the levels of ARC expression in CMM and PVN (given the

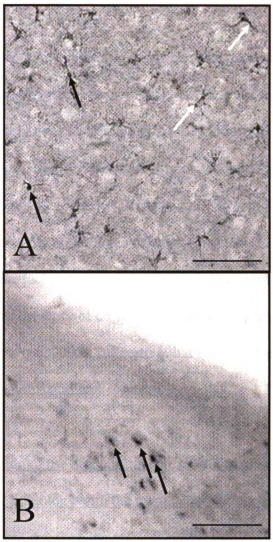

Figure 7.2. Expression of the protein product of the immediate early gene ARC in NCM (A) and the dorsomedial hippocampus (B). Labeled protein was observed in nuclei (black arrows) and dendrites (white arrows). Scale bar = 15 μm (A) and 50 μm (B).

reciprocal connections between CMM and NCM (Chapter 1) and between the PVN and hippocampus (Chapter 5)) to more fully understand the changes that occur in regions important for song behavior in females.

The expression of immediate early genes can also depend on a bird's immediate environment, specifically the presence or absence of other birds. Adult songbirds show little to no immediate early gene expression in Area X following song exposure (Jarvis & Nottebohm, 1997; Jarvis, Scharff, Grossman, Ramos, & Nottebohm, 1998; Jin & Clayton, 1997) or when a male sings to a female. However, ZENK is induced in this region when males sing in the presence of another male or alone (Jarvis et al., 1998). Additionally, levels of ZENK immunoreactivity are greater in the male zebra finch NCM when female calls are presented in the presence of other males than when presented in social isolation (Vignal, Andru, & Mathevon, 2005), suggesting that social context modulates immediate early gene activity within this auditory region.

The structure and function of the hippocampus in zebra finches are similar to those in a non-songbird and show homology with the mammalian hippocampus

Together with data detailing the efferent connectivity of subdivisions of the zebra finch hippocampus (Székely & Krebs, 1996), data from Chapter 5 indicate similarity with the intrahippocampal circuitry of the pigeon hippocampus, and both are reminiscent of the trisynaptic pathway in the mammalian hippocampus (Figure 7.3). This suggests a similar means by which the multisynaptic, feedforward excitation of the mammalian hippocampus by stimulation of the entorhinal cortex (Yeckel & Berger, 1990) can also occur in the avian hippocampus. Based on these anatomical similarities and the

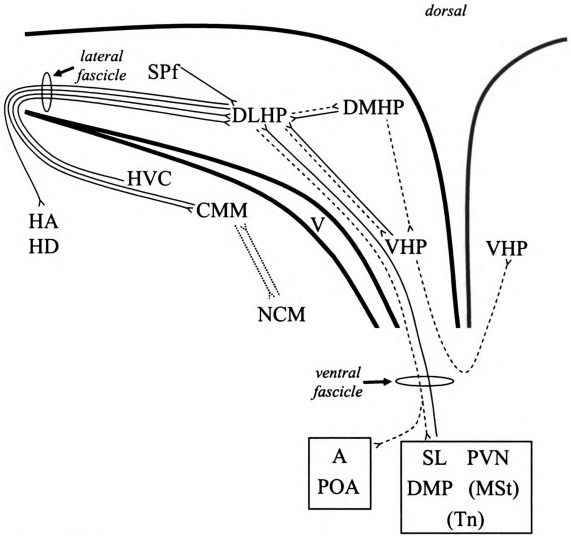

Figure 7.3. Intrahippocampal and intertelencephalic connections of subdivisions of the zebra finch hippocampus compiled from Székely and Krebs (1996; dashed lines) and data I have collected (Chapter 5; solid lines), detailing potential involvement with the song system. A known connection between CMM and NCM is indicated by a dotted line; see Figure 1.1 (Chapter 1) for additional connections among song control regions. A = arcopallium, CMM = caudomedial mesopallium, DLHP = dorsolateral subdivision of the hippocampus, DMHP = dorsomedial subdivision of the hippocampus, DMP = posterior portion of the dorsomedial thalamic nucleus, HA = hyperpallium apicale, HD = hyperpallium densocellulare, MSt = medial striatum, NCM = caudomedial nidopallium, SL = lateral septum, SPf = Substance P reactive nucleus, POA = preoptic area, PVN = periventricular nucleus, Tn = nucleus taeniae, V = lateral ventricle, VHP = ventral subdivision of the hippocampus. Parentheses enclose structures in which retrogradely labeled cells were observed in only one bird.

functional similarities detailed in Chapter 6, the hippocampus in zebra finches appears to be a functional homologue of that in mammals.

Zebra finches are an ideal model system for the further examination of physiological processes underlying specific forms of learning and memory

Given the song-specific induction of immediate early genes in the hippocampus (Chapters 3 and 4), its connections with regions involved in song behavior, motivation and arousal (Chapter 5), and the effects on performance in a spatial memory task following lesions of a subdivision of the region (Chapter 6), the hippocampus in male and female zebra finches is an excellent model with which to study the physiological underpinnings of learned behavior. Along with NCM, the hippocampus of adult male and female zebra finches is high in aromatase (Saldanha, Popper, Micevych, & Schlinger, 1998), the enzyme responsible for estrogen synthesis, and estrogen is important for various forms of learning and increased cognitive function (McEwen & Alves, 1999; Oberlander et al., 2004; Rissman, Heck, Leonard, Shupnik, & Gustafsson, 2002). Aromatase levels remain high in the hippocampus from just before the critical period for song learning through adulthood (Jacobs, Arnold, & Campagnoni, 1999). Estrogen is important in song learning in juvenile male zebra finches: castration and systemic treatment with the estrogen antagonist tamoxifen results in the production of abnormal song (Bottjer & Hewer, 1992). It is reasonable to hypothesize that hippocampal estrogen specifically is involved. Estrogen receptors are present in the avian hippocampus (Metzdorf, Gahr, & Fusani, 1999), so the hormone could act locally, or a high rate of

hippocampal estrogen production could allow for diffusion to other telencephalic regions important for song production or perception.

Thus, perhaps lesions of only the dorsolateral subdivision (Chapter 6) may not have been enough to disrupt normal song behavior because aromatase availability (and thus estrogen production), for one, in other regions was enough to compensate for the loss of only 10-20% of the region. In addition, aromatase mRNA and protein are increased following damage to the brain in zebra finches (Peterson, Saldanha, & Schlinger, 2001), which could also compensate. Interestingly, aromatase and NMDA receptors are coexpressed in neurons of the hippocampus (Saldanha et al., 2004). Estrogen treatment increases the innervation of NMDA receptor-positive neurons and the density of presynaptic NMDA autoreceptors. As indicated above, glutamatergic receptors are integral to LTP (Nordeen & Nordeen, 2004) and song learning (Aamodt et al., 1996; Basham et al., 1996; Heinrich et al., 2002; Nordeen & Nordeen, 2004; Scott et al., 2004; Singh et al., 2000). So, if estrogen synthesis in the hippocampus is important for the function of the song system in either sex, then perhaps its inhibition will produce deficits. A study like this would provide additional insight into the function of the hippocampus in song behavior specifically and, more generally, a potential physiological dissociation of the nature of memory encoding by the hippocampus.

General Summary

Studies in the zebra finch have focused primarily on the development of and neural mechanisms involved in male song learning and production and, in association, the sexual differentiation of brain and behavior. The studies of this dissertation have

furthered our knowledge regarding the development of neural responses to song in both males and females, the regions and mechanisms involved, and have begun to elucidate additional neuronal circuits and structures in the sexes that may modulate song memory formation and expression.

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