

Hippocampal Lesions Impair Spatial Memory Performance, but Not Song – A Developmental Study of Independent Memory Systems in the Zebra Finch

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Received 12 November 2008; revised 19 January 2009; accepted 7 February 2009

ABSTRACT: Songbirds demonstrate song- and spatial-learning, forms of memory that appear distinct in formal characteristics and fitting the descriptions and criteria of procedural and episodic-like memory function, respectively. As in other vertebrates, the neural pathways underlying these forms of memory may also be dissociable, and include the corresponding song circuit and hippocampus (HP). Whether (or not) these two memory systems interact is unknown. Interestingly, the HP distinguishes itself as a site of immediate early gene expression in response to song and as a site of estrogen synthesis, a steroid involved in song learning. Thus, an interaction between these memory systems and their anatomical substrates appears reasonable to hypothesize, particularly during development. To test this idea, juvenile male or female zebra finches received chemical lesions of the HP at various points during song learning,

as did adults. Song structure, singing behavior, song preference, and spatial memory were tested in adulthood. Although lesions of the HP severely compromised HP-dependent spatial memory function across all ages and in both sexes, we were unable to detect any effects of HP lesions on song learning, singing, or song structure in males. Interestingly, females lesioned as adults, but not as juveniles, did lose the characteristic preference for their father's song. Since compromise of the neural circuits that subserve episodic-like memory does very little (if anything) to affect procedural-like (song learning) memory, we conclude that these memory systems and their anatomical substrates are well dissociated in the developing male zebra finch. © 2009 Wiley

Periodicals, Inc. *Develop Neurobiol* 00: 000–000, 2009

Keywords: hippocampus; ibotenic acid; aromatase; immediate early gene; estrogen; songbird

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Contract grant sponsor: NIH; contract grant numbers: RO1 MH55488, K02 065907, F31 MH64982, RO1 NS 042767.

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Published online in Wiley InterScience(www.interscience.wiley.com). DOI 10.1002/dneu.20713

INTRODUCTION

The adult vertebrate demonstrates two dissociable memory systems, defined often as implicit and explicit memory function. Examples of the type of behaviors these memory systems subserve include, but are not limited to, “habit or skill formation” and memory for “what, when, and where.” Studies on

humans, nonhuman primates, and rodents strongly suggest a distinction in the neural pathways that underlie these types of procedural and episodic memories. Although the basal ganglia and striatum are critical players in procedural memory function (Barnes et al., 2005), the hippocampus (HP) seems to be crucial for veridical episodic memory, although not exclusively (Eichenbaum, 2004). Clinical examinations have also revealed that disruptions in one form of memory typically do not interfere with performance on the other (Squire, 2004). Thus, at the structural, behavioral, and clinical levels, procedural and episodic memory function appear dissociated.

Songbirds (Order: Passeriformes) also demonstrate evidence of procedural and episodic memory function. Decades of behavioral research on song learning strongly suggest that it is a learned sensorimotor skill (Marler, 1997). Additionally, the neural pathways necessary for song learning involve areas with striatal and pallial characteristics such as Area X and the medial and lateral portions of the magnocellular nucleus of the anterior nidopallium (m- and lMAN; Bottjer et al., 1984; Scharff and Nottebohm, 1991). In adult songbirds, nuclei essential to song expression have been determined by lesion studies, electrophysiological analyses, and by examination of immediate early gene (IEG) expression. Neuronal activation is observed in the sensorimotor integrative nucleus HVC and the robust nucleus of the arcopallium (RA; Jarvis and Nottebohm, 1997; Margoliash, 1997), and lesions to these telencephalic nuclei during song learning or adulthood disrupt procedural memory-based song output (Simpson and Vicario, 1990; Scharff and Nottebohm, 1991). More recent studies also reveal that some songbirds show compelling evidence of an episodic memory system including memory of what, where, and when (Clayton and Dickinson, 1998; Griffiths et al., 1999; Clayton et al., 2003, 2007). Lesions of the HP severely impair what may be forms of this memory function. Specifically, in food-storing songbirds such as the black-capped chickadee (*Peocile atricapillus*), HP lesions decrease memory for locations containing stored food but do not impair the motivation to store (Sherry and Vaccarino, 1989), and food-storing songbirds have a bigger HP relative to the rest of the brain compared to closely related nonstorsers (Krebs et al., 1989). HP lesions also affect spatial memory performance in zebra finches (*Taeniopygia guttata*; Patel et al., 1997a; Watanabe and Bischof, 2004), and acquisition of a spatial memory task is associated with greater cell size in the anterior HP in these birds (Oberlander et al., 2004).

Hence, at the levels of behavior and neuroanatomy, the procedural-like memory involved in song learning and the episodic-like memory involved in spatial memory function appear dissociated, although this hypothesis awaits rigorous testing (see later). Interestingly, IEG responses to conspecific song stimuli are detected in HP neurons (Bailey et al., 2002; Bailey and Wade, 2003, 2005) in patterns that mirror those in auditory perceptual regions (Mello and Clayton, 1994) that are afferent to the aforementioned structures of the procedural memory circuit (Vates et al., 1996; Mello et al., 1998). In fact, a number of studies report the expression of IEGs or their induced cascades in the HP in response to auditory stimulation with novel conspecific song or tutor song stimuli (Kimpo and Doupe, 1997; Bolhuis et al., 2000, 2001; Eda-Fujiwara et al., 2003; Cheng and Clayton, 2004) or when conspecific song is paired with a novel visual stimulus (Kruse et al., 2004). To the best of our knowledge, there are no documented direct connections between the HP and any of the song nuclei, or direct connections with perceptual areas, such as the caudomedial nidopallium (NCM). However, the HP may communicate with the dorsomedial portion of the thalamic nucleus (Székely and Krebs, 1996), part of a “thalamo-cortical” circuit possibly involved in song learning or sensorimotor integration (Vates et al., 1997), as well as portions of the arcopallium, perhaps including neurons associated with RA or its “cup” (Székely and Krebs, 1996), and the medial striatum, which expresses genes important to song learning and human speech development (Teramitsu et al., 2004). Connections of the HP with areas that may be involved in song-related behavior also exist, including the nucleus taeniae, which is involved in the control of sexual behavior in Japanese quail (Absil et al., 2002), and the septum, which is implicated in spatial memory and motivated behaviors in several avian species (Shiflett et al., 2002).

At a very general level, previous work suggests a specialization of the songbird HP. Specifically, aromatase, the enzyme responsible for estrogen synthesis, is abundantly expressed in the HP of every passerine species studied (Saldanha and Schlinger, 1997; Foidart et al., 1998; Saldanha et al., 1998, 1999; Soma et al., 2003). In stark contrast, this enzyme is low to undetectable in the nonpasserine HP (Hutchison and Steimer, 1984; Schumacher and Balthazart, 1987; Balthazart, 1990; Saldanha et al., 1998). Given the important role of estrogen in song learning (Marler et al., 1988), this correlation raises questions about a possible role for the HP in the modulation of some aspect of singing behavior.

The song-specific responses and neurochemical makeup of the HP, and that disruption of HP circuits compromises spatial memory function in several species, permits the testing of a tantalizing question: are the neural substrates for episodic and procedural learning associated during development? We began to address this question by lesioning the HP of juvenile (20 (d20) or 45 (d45) days posthatch) male and female zebra finches, just as they entered the template formation and sensorimotor integration phases, respectively (as defined in males; Nordeen and Nordeen, 1997). Adult male and female zebra finches were also either lesioned or sham lesioned. All subjects were tested on spatial memory function, song learning (mimicry of tutor), and song preference (preference for tutor and conspecific songs). Decrements in song production or song perception, concurrent with attenuated spatial memory ability, would indicate an integrated role for the HP in the development or utilization of song-related memories.

MATERIALS AND METHODS

Animals and Housing

Birds housed in breeding colonies at Michigan State University (juveniles and adults) and Lehigh University (adults only) were used in the experiments. Birds from both institutions were initially housed in communal aviaries, maintained on a 12:12 light:dark cycle (lights on at 0700 h), and 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 or after (birds tested at Lehigh were all greater than 100 days of age, although their precise ages were not known) and placed in individual stainless steel cages (11" × 9" × 12") with free access to water, gravel, and seed. While in individual cages, birds continued to have visual and auditory contact with conspecifics.

At Michigan State, 89 birds from the breeding colony were used. Of those, 15 died following surgery or shortly thereafter, 12 had lesions ventral to the HP, 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 (details of the test are given later) and were removed from the experiment. The number of birds remaining for the groups was as follows, equally divided between lesion and sham lesion, with one exception: d20, $n = 10$ males and 9 (five lesion, four sham) females; d45, $n = 10$ males and 10 females; and d100, $n = 10$ males and 10 females. Three males and two females were used from the breeding colony at Lehigh University for validation of the spatial memory test only. Only birds at Michigan State were lesioned/sham lesioned, and all statistical analyses reported later are from those birds.

Lesion Procedure

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 into the electrode holder of a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). Birds were deeply anesthetized with isoflurane and mounted in the stereotaxic frame with their head angled at 45° (Stokes et al., 1974). The skull was exposed, the intersection of the lambdoidal and sagittal sutures measured, as were relative coordinates for injections into the HP (anterior/posterior = +1.0, lateral = ± 0.5 relative to the intersection point). Bilateral pressure injections of 0.1 μL of 10 mg/mL ibotenic acid (from Patel et al., 1997a; 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 HP, aimed at the dorso-lateral subdivision of the structure where conspecific song-induced IEG activation was previously detected (Bailey et al., 2002; Bailey and Wade, 2003, 2005). In d45 and adult birds, holes were drilled through the skull using a 1-mm diameter engraving cutter in a Dremel[®] rotary tool (S-B Power Tool Co., Racine, WI), and the micropipettes were lowered 0.3 mm relative to the dura. No holes were drilled in the skulls of d20 zebra finches; the micropipettes were pierced through the thin, flexible skull and lowered to a ventral depth of 0.55 mm relative to the skull's surface to account for its 250–300 μm thickness. Micropipettes remained in the brain for 1 min following the pressure injection to ensure diffusion of the ibotenic acid or vehicle.

Craniotomies were covered with bone wax (Fine Science Tools Inc., Foster City, CA), skin flaps reconnected with silk suture (Ethicon, Inc., Somerville, NJ) and sealed with collodion (Mallinckrodt Baker, Inc., Phillipsburg, NJ), and birds were removed from the stereotaxic frame and placed on a heating pad. Because zebra finches need parental care for feeding until \sim d35, and because song learning in the presence of a tutor begins around d25 and continues through \sim d65, birds lesioned at d20 and d45 were returned to their home cages following surgery and recovery. Birds lesioned as adults were returned to the colony in an individual cage, where they remained throughout testing.

Since all behavioral tests (except those for song production in adult males before the lesion, noted below) were done in the same room, the spatial memory test was run first to ensure the novelty and therefore the HP dependency of the task (Squire, 2004). 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 counter-balanced and separated by intervals of \sim 24 h.

Spatial Memory Test

A modified "t-maze" was designed to examine spatial memory in zebra finches, initially at Michigan State University. The apparatus (Fig. 1) consisted of a wood frame

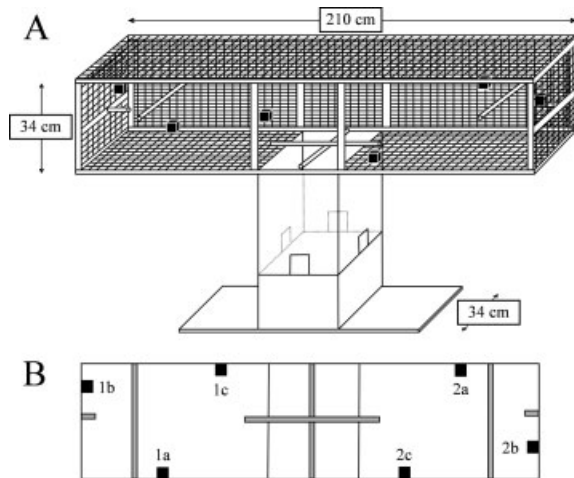


Figure 1 (A) Chamber used for spatial memory tests following HP lesions. Doors at the bottom of the chamber were used as entrance points. (B) Chamber diagrammed from above. Perches (gray) at the ends of the chamber that run parallel with the front of the maze sat 16 cm above the floor of the arms, and those that run perpendicular in each arm were 20 cm above. In the middle of the chamber were perches in the shape of a plus, with a portion extending 5 cm into each arm, 3 cm above the floor of each. The plastic cups (2 cm × 2 cm × 3 cm) were 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.

and steel mesh on all sides so extra-maze cues could be observed from inside the apparatus and through which behavior could be viewed and recorded. 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.

Prior to testing, birds were food deprived at lights on for ~4–5 h (see Oberlander et al., 2004), following which each bird underwent acquisition trials. The bird was placed in the chamber through the door in the bottom of the column. The flaps of all cups were open, and only one was filled with seed. Pilot work determined that the vast majority of birds took 20 min or less to acclimate to the chamber and eat from the baited cup (data not shown). Thus, birds were given 20 min (maximum latency) to explore the chamber and locate the seed. When a bird located the baited cup within this 20-min period it was given 30 s to eat, after which the lights were turned off, and the bird was removed and returned to its cage in a separate room. Every 10 min, the bird was placed back into the chamber through the same door until the arm that contained food was entered within 30 s on three consecutive trials (criterion). Upon reaching criterion, birds were returned to their individual cage for 1 h.

Following this 1-h period, birds underwent probe trials, separated by 10 min, to determine their memory for the

location of the baited cup. Cups that did not contain seed were filled and then emptied to eliminate any olfactory cues birds used to locate the seed. 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 recorded as “examined” if 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 s 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.

To confirm the reliability and validity of this novel test of spatial memory, especially in light of inconsistencies that can occur in the results of behavioral tests between laboratories (e.g., Crabbe et al., 1999), an identical apparatus was built for testing at Lehigh University, but the procedure was varied slightly. Birds were removed from communal aviaries and placed into individual cages 1 week prior to testing. On the day of testing, a bird was food deprived and tested for acquisition as mentioned earlier, but only one probe trial was run, and no seed was kept in the baited cup during that trial to confirm that a memory for the cup’s location, not visual or olfactory stimuli, was used by the birds. A cup was recorded as “visited” if a bird leaned toward it while perched near or by it, or if its flap was lifted as mentioned earlier. These data were not analyzed with those collected from birds tested at Michigan State.

Song Preference Tests

Preference for the song of a tutor versus that of a novel male conspecific, as well as novel male conspecific versus heterospecific song, was tested in an apparatus (215 cm in length, 60 cm in height, 60 cm deep) 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.

Birds were placed in the middle of the chamber through a door in the Plexiglas and allowed to acclimate for 1 h. At this point, the songs of two zebra finch males (one from the father and a 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. Songs were broadcast synchronously from speakers at each end of the apparatus [“approach zones”; similar to procedures used by Clayton (1988) and Miller (1979)]. The middle of the apparatus between the two ends was considered a “neutral zone” (Clayton, 1988). 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 to eliminate the preference for a “zone” of the chamber that may be conferred by a prior 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 for analysis. Difference scores for each bird were obtained by subtracting the amount of time spent in the side of the chamber broadcasting father’s song minus that of another conspecific, as well as zebra finch song minus heterospecific song. To determine whether age or sex at the time of HP lesion differentially affected these preference measures, data were analyzed between the sham and lesion groups within each treatment age and sex by ANOVA.

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 to compare to the songs of their male offspring (except for birds lesioned as adults) in the determination of whether HP lesions affected song learning, and as one of two simultaneously played conspecific songs in tests of song preference as indicated above. 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 using Windows Movie Maker (version 2.1.4026.0). Audio was extracted from the video files using Adobe Audition (version 1.0).

All song was recorded when experimental birds were adults. To determine whether HP lesions affected song production or stability in adulthood, songs prelesion (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 (range = 7–10 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). Thus, to determine whether HP lesions affect the ability to match 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 ~152 days post-hatch, and those lesioned at d45 at about 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 postmanipulation 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 et al., 2001). To eliminate any subjective bias that may have led to unreliable determinations of song similarity, Sound Analysis Pro software was used (Tchernichovski et al., 2000) to measure and compare the features of the collected songs. Phrases were picked from each song based on the “sharpness” of the phrase’s spectral derivative (as computed by Sound Analysis Pro) and if no extraneous noise (wing flapping, calling by the female, etc.) could be heard in the phrase. Similarity scores of pupil versus tutor phrases (for birds lesioned as juveniles) and phrases before versus after lesion (for birds lesioned as adults) were obtained. The overall similarity score calculated by Sound Analysis Pro is an aggregate of the percent similarity, mean accuracy, and sequential match of notes calculated within and between particular phrases.

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 the 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 transcardially with PBS followed by 4% paraformaldehyde in PBS. Brains were postfixed for 1 h in paraformaldehyde, sunk overnight in 30% sucrose in PBS, cut frozen on a cryostat into 30- μ m coronal sections onto Superfrost Plus slides (Fisher), and stained with thionin. The HP of each lesioned bird was reconstructed using NeuroLucida software (version 6.02.1, MicroBrightField, Inc.). Contours were traced in every fourth section around the border of the HP and the area within the structure in which cell bodies were destroyed, to confirm the accuracy and extent of the HP lesion. Total HP 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 and from which data were collected.

RESULTS

Histology

The extent of HP tissue damage (Fig. 2) induced by the excitotoxin ranged from approximately 10–23% of the structure (mean percent damage to the HP of birds lesioned as adults: 17.55 \pm 1.36; d20: 13.40 \pm 0.92; d45: 15.00 \pm 1.06), confined mostly to the

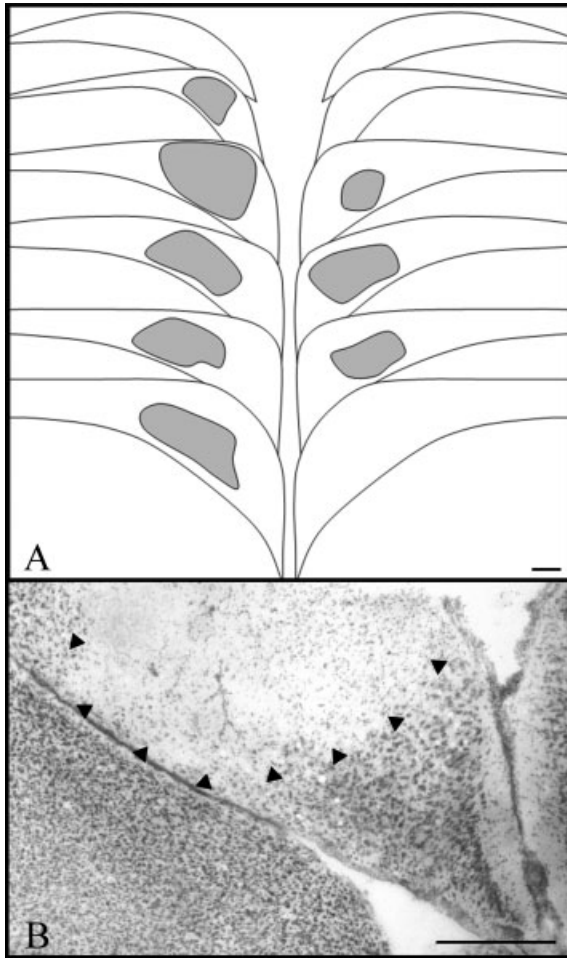


Figure 2 (A) Reconstruction of a lesion (gray) through rostral (bottom) to caudal (top) sections of the HP in a male zebra finch (lesion size relative to HP volume in this bird was 17.52%) injected with ibotenic acid as an adult, reproduced from tracings obtained via NeuroLucida software (see Methods). (B) Coronal section stained with thionin at the level of the hippocampus detailing areas of cell death (indicated by arrows) induced by the lesion. Scale bars in both panels = 200 μm .

dorsolateral subdivision. Birds with lesions lateral or ventral to this location were excluded from the analysis below. The amount of damage depending on age at time of lesion approached statistical significance [$F(2, 24) = 3.15, p = 0.061$], 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. Lesion size was not dependent on sex [$F(1, 24) = 0.03, p = 0.869$] nor was there an interaction between age and sex [$F(2, 24) = 0.36, p = 0.704$] on the extent of the lesion.

Developmental Neurobiology

Spatial Memory

Birds with lesions of the HP did not significantly differ from their sham controls in the time spent to initially eat from the uncovered, baited cup during the first acquisition trial (mean = 664.69 ± 38.92 s; all $F < 1.61$, all $p > 0.211$ for main effects of age, sex, treatment group and interactions) or in the number of trials needed to reach the criterion level during acquisition (mean = 5.32 ± 0.13 trials; all $F < 1.74$, all $p > 0.187$).

However, the amount of time taken to eat from the covered, baited cup during the four probe trials was significantly different between lesioned and sham-lesioned birds [Fig. 3; $F(2, 141) = 9.83, p = 0.003$], and these latencies significantly decreased over the course of the probe trials [$F(3, 141) = 37.81, p < 0.0001$]. Further, the times of males to eat from the baited cup in both the sham and lesion groups decreased more abruptly over the probe trials than did those of females [$F(3, 141) = 2.95, p = 0.035$; data not shown]. No other main effects or two-, three-, or four-way interactions were significant (all $F < 1.95$, all $p > 0.077$).

Over the four probe trials, sham-lesioned birds spent a significantly larger percentage of time in the arm that contained the baited cup [$F(1, 141) = 26.02, p < 0.0001$]. It is expected that birds without a memory for the location of the seed would spend, on average, equal time searching in both arms. Lesioned birds spent $\sim 59\%$ in the goal arm during the first probe trial, while sham-lesioned birds spent 77%. Over the probe trials, the amount of time spent in the

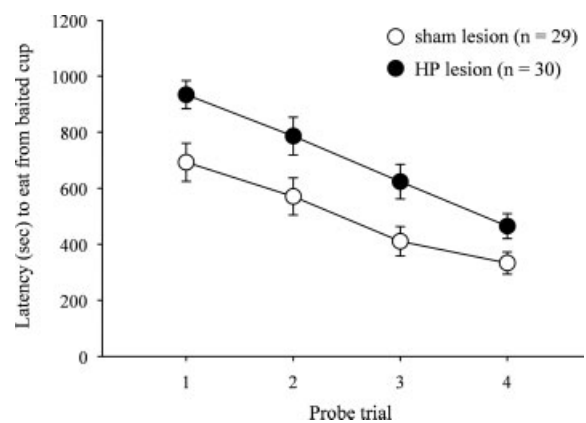


Figure 3 Mean (\pm SEM) latencies to eat from the baited cup across the four probe trials for all lesioned and sham-lesioned birds. As a group, lesioned birds took significantly longer than sham-lesioned birds to eat from the baited cup, yet both groups showed a significant decrease in these latencies over the course of the probe trials.

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 striking 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 errors than the sham controls [$F(1, 141) = 64.52$, $p < 0.0001$]. The number of mistakes was affected by age at time of lesion [$F(2, 141) = 5.03$, $p = 0.011$]. Errors made by females tended to be greater, although not significantly, than those by males [$F(1, 141) = 3.70$, $p = 0.060$]. Mistakes decreased over the course of the probe trials for all birds [$F(3, 141) = 4.18$, $p = 0.007$] but at different rates for the lesioned and sham-lesioned birds [$F(3, 141) = 6.94$, $p = 0.0002$]; mistakes by sham-lesioned birds remained generally steady over the probe trials, but decreased by more than half for lesioned animals. The three-way interaction of age, sex and treatment also approached significance [$F(6, 141) = 2.03$, $p = 0.065$]. No other interactions were significant (all $F < 2.29$, all $p > 0.112$).

Given the main effects and interactions indicated earlier, mistakes over the course of the probe trials were analyzed further. Examining the first probe trial (Fig. 4), lesioned birds made significantly 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 [$F(1, 47) = 22.96$, $p < 0.0001$; Fig. 4], an effect that depended on age at the time of lesion [$F(2, 47) = 3.64$, $p = 0.039$]. *Post hoc* tests revealed that birds lesioned/sham lesioned as adults 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, females appeared to make slightly 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-way interactions were significant (all $F < 2.52$, all $p > 0.091$). Analysis of mistakes made by sham birds revealed that control females made more mistakes than control males [$F(1, 23) = 7.98$, $p = 0.0075$]. 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 this probe trial 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 [$F(1, 47) = 47.94$, $p < 0.0001$; Fig. 4]. Birds lesioned at d20 made fewer mistakes than birds in 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 again 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 [$F(1, 47) = 5.77$, $p = 0.021$; Fig. 4]. No other significant effects or interactions were uncovered when all animals were analyzed collectively or when control or lesioned birds were examined alone (all $F < 2.04$, all $p > 0.147$).

As mentioned earlier, five additional birds were tested in a reconstruction of the spatial memory apparatus at Lehigh. The mean initial latency to eat from the baited cup in the acquisition phase by the three male and two female birds was 906.80 (± 123.25) s, and the trials to criterion was 5.20 (± 0.37). During the one-probe trial, the Lehigh birds "examined" or lifted the flaps of cups an average of 6.80 (± 1.24) times, with 90.0% of those directed toward the baited cup, and these five birds spent 86.3% of their time in the arm that contained the baited cup.

Song Preference

Lesions of the HP did not affect birds' collective normal preferences for tutor song over the song of a novel male zebra finch (Fig. 5). Preferences were not affected by age at the time of lesion [$F(2, 47) = 0.74$, $p = 0.485$], sex [$F(1, 47) = 0.01$, $p = 0.933$] or treatment group [$F(1, 47) = 0.48$, $p = 0.494$], and no significant interactions were found between any of these

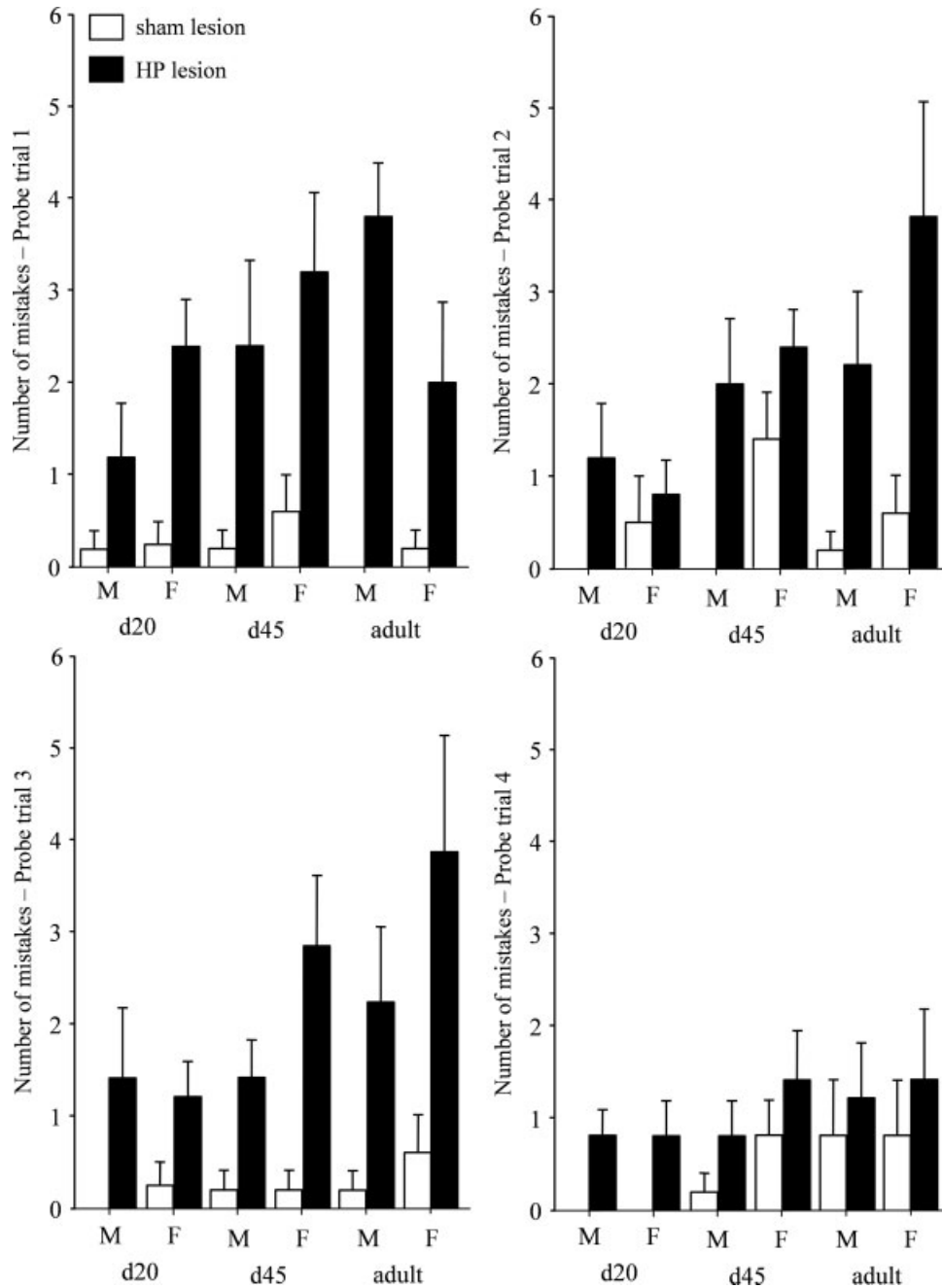


Figure 4 Mean (\pm SEM) number of incorrect cups examined or flaps of those cups lifted in the four probe trials by adult male (M) and female (F) zebra finches lesioned or sham lesioned at d20, d45, or in adulthood. Overall, lesioned birds made significantly more mistakes than sham-lesioned ones, and birds lesioned at younger ages made fewer mistakes than birds lesioned as adults. Mistakes by sham-lesioned birds remained generally steady over the course of the probe trials, while those by HP-lesioned birds decreased by more than half.

variables (all $F < 1.06$, all $p > 0.356$). Only one group (females lesioned in adulthood) showed no preference for their tutor compared to a novel male, based on the difference in times spent in each half of

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the chamber (Fig. 5); no additional statistical analyses were performed given the lack of main effects.

Preference for novel conspecific song over the song of a heterospecific (Fig. 6) was unaffected by

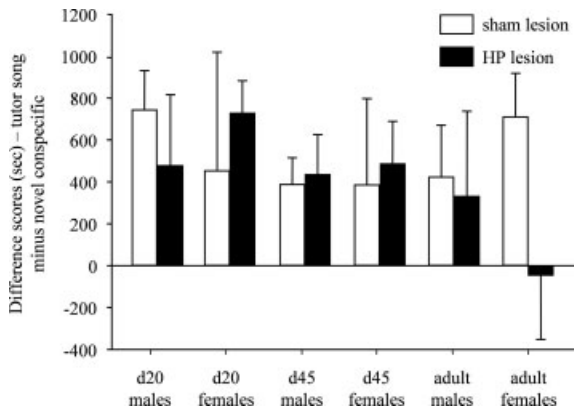


Figure 5 Mean (\pm SEM) difference scores (amount of time (sec) spent in side of chamber playing father's song minus amount of time spent in side of novel male conspecific song) of male and female zebra finches HP- or sham-lesioned at 20 days posthatch (d20), d45, or adulthood. A positive score indicates 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.

lesions of the HP [$F(1, 47) = 0.00, p = 0.961$], nor was it 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

Lesions of the dorsolateral HP in adult males did not affect their ability to produce song as compared pre- and postsurgery. 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 comparisons of select phrases from songs recorded

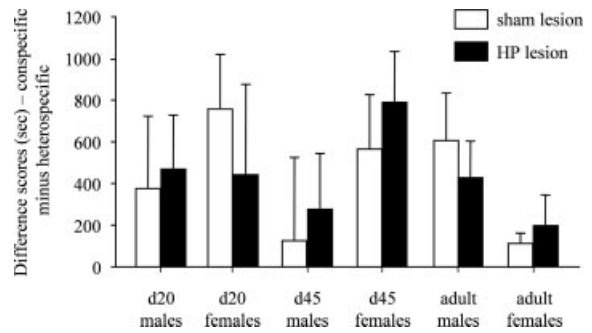


Figure 6 Mean (\pm SEM) difference scores (amount of time (sec) spent in side of chamber playing conspecific song minus amount of time spent in side of heterospecific song) of male and female zebra finches HP- or sham-lesioned at 20 days posthatch (d20), d45, or adulthood. No significant main effects or interactions were uncovered.

pre- and postsham or lesion (Table 1). Qualitatively, no additional notes or changes in note order were observed in the sonagrams of any of the adult-lesioned birds (Fig. 7).

Overall similarity scores of birds lesioned at d20 or d45 did not significantly differ [Table 1; main effect of age: $F(1, 16) = 1.47, p = 0.243$; treatment: $F(1, 16) = 1.30, p = 0.271$]. Percent similarity with a tutor's song phrase was not influenced by age of lesion [$F(1, 16) = 0.27, p = 0.613$] but was, interestingly, higher in the lesioned animals compared to the sham controls [$F(1, 16) = 6.29, p = 0.023$]. The mean accuracy and sequential matches between tutor and pupil's song phrases were not significantly affected by the hippocampal lesion [$F(1, 16) = 0.79, p = 0.387$ and $F(1, 16) = 0.004, p = 0.952$] nor by the age at time of lesion [$F(1, 16) = 0.17, p = 0.689$ and $F(1, 16) = 1.10, p = 0.310$, respectively]. No significant interaction between age and group was uncovered through analysis of any combination of the song similarity measures (all $F < 3.73$, all $p > 0.071$).

Table 1 Similarity Measurements of Song Phrases from Adult Male Zebra Finches Lesioned or Sham-Lesioned at d20, d45 or in Adulthood

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

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.

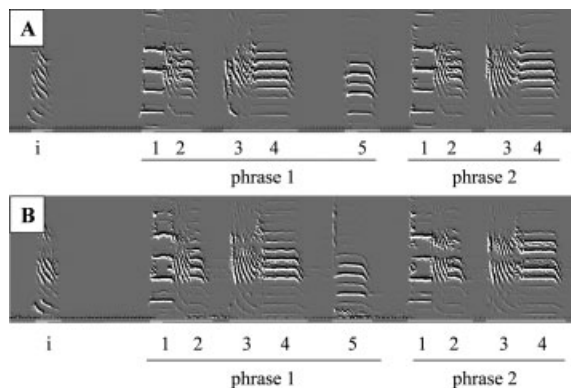


Figure 7 Spectral derivatives of samples of the songs of a male zebra finch before (A) and after (B) a bilateral lesion of the hippocampus. Note that the types of notes used and the order of those notes are identical before versus after the lesion.

DISCUSSION

The roles of the passerine HP and the song circuit in spatial memory function and song learning, respectively, are well established. The HP plays a pivotal role in spatial memory function in several passerines (Sherry and Vaccarino, 1989; Hampton and Shettleworth, 1996; Patel et al., 1997a,b; Oberlander et al., 2004; Watanabe and Bischof, 2004). Correspondingly, over 30 years of research has unraveled the importance of various song nuclei and associated pathways in song learning, production, and perception (Bolhuis and Gahr, 2006). The anatomical disjunction between the pathways that subserve these forms of memory function provides an excellent model to test their interdependence during development but specifically the potential role of the HP in song behavior. The current set of experiments strongly suggest that HP lesions during the period of song learning, while dramatically decreasing spatial memory performance tested in adulthood, have no detectable effects on various aspects of song learning, including song production, song structure, and song preference in adulthood. These results suggest that IEG activity (Bailey et al., 2002; Bailey and Wade, 2003, 2005) in the HP following stimulation with conspecific song does not confer a role for the region in song learning or perception *per se*, but do not exclude the possibility that the structure is involved in the acquisition of a conditioned place preference. For example, this might include a memory relating to the environment (conditioned stimulus) in which songs (unconditioned stimuli) are heard or produced, rather than the song itself.

Effects of HP Lesions on Spatial Memory

The current findings extend previous experimental findings and interpretations in several ways. First, we specifically tested the role of HP lesions in songbirds during development on spatial memory performance as adults. Lesions occurring up to 80 days prior to testing continued to adversely affect spatial memory performance, and this effect is due to the destruction of neurons with cell bodies resident within the HP and not fibers of passage. Second, it is possible that birds in HP lesion groups used alternate strategies to determine the location of the food source outside of the realm of spatial processing, as HP-lesioned birds in a prior study (Watanabe and Bischof, 2004). The performance of birds in the current experiment improved over the course of the probe trials, indicated by 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. 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 and the relatively confined area of HP damage were 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 h. Third, the performance of birds lesioned at d20 was better than adults on a few measures. Perhaps correspondingly, the amount of HP damage was lower (although not statistically different) in birds lesioned at d20 than those lesioned in adulthood. It is possible that neurogenesis occurred in the region in the months following the lesion, as damage to the HP induces upregulation of neuroprotective estrogen to new neurons (Peterson et al., 2004) and that environmental change induces neurogenesis in other brain nuclei, such as HVC and Area X (Lipkind et al., 2002). Given the increased performance of these birds, their smaller lesion volume, that neurogenesis within the avian brain (Patel et al., 1997b; Nottebohm, 2002) is well documented, and that the HP in mammals is a primary site for functional neurogenesis (van Praag et al., 2002), this is a possibility. Fourth, females made significantly 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 probe session. Males of many species show consistent advantages in a variety of spatial tasks (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 were seen. Questions remain as to whether the morphology, neurochemistry or electrophysiological activity of the HP differs between the sexes in the zebra finch, or whether differences in nonspatial learning tasks exist. Finally, the spatial memory apparatus developed appears to be a reliable and valid means with which to detect spatial memory behavior and attenuation in it in a nonfood storing bird like the zebra finch, and the results show that these birds do not use olfactory cues to locate the seed. Taken together, these findings add to the growing body of literature that supports a pivotal role for the avian HP in spatial memory function during adulthood and now during development as well.

Lack of Effects of HP Lesions on Song Learning, Perception, or Production

The neural pathways responsible for song learning and spatial memory acquisition are documented. Several years of neuroanatomical studies have clearly established the afferents, efferents, and collateral projections of neurons that collectively form the song circuit in songbirds (Wild, 2004). Fewer studies have examined the anatomical connectivity of the passerine HP (Berk and Hawkin, 1985; Székely and Krebs, 1996; Székely, 1999; Atoji and Wild, 2004, 2006). Even a casual glance at the inputs and outputs of the HP and the sensorimotor integrative nucleus HVC reveals a striking lack of overlap in the circuits mediating spatial memory function (HP) and song (HVC). Functionally, neurons within HVC are integral to song behavior, as destruction of neurons within this nucleus, in development or adulthood, disrupts song output (Simpson and Vicario, 1990; Scharff and Nottebohm, 1991), and these cells incorporate into the nucleus and motor pathway during the song learning period (Nordeen and Nordeen, 1988). The present results indicate that cells within the HP do not influence song learning or production as in this “traditional” song control nucleus.

Lesions of the HP at d20 and d45 did not disrupt formation of a song template, the later sensorimotor integration required for song stabilization, or normal song preference in adulthood. HP lesions during adulthood had no effect on male singing behavior, song structure, or song preference. In females, normal song preferences for conspecific over heterospecific song were maintained following HP lesions, unlike, for example, following lesions of the caudomedial

mesopallium (CMM; MacDougall-Shackleton et al., 1998). However, we were struck by the loss of preference for father’s song demonstrated by females who received HP lesions as adults. This finding is tantalizing given the potential role of the HP in episodic-like characteristics of song perception. Specifically, the identity (individual bird), orientation (spatial location), and temporal context (time of day and/or year) of incident song may likely involve HP pathways (Marler, 1997). However, this finding must be put in the context of other proceptive behaviors performed by adult zebra finch females in response of song. More work is needed to unravel this exciting, potential role for the HP in song perception in songbirds. Nevertheless, the overwhelming conclusion from these data is that the HP does not appear to influence several aspects of song behavior in the zebra finch, and, in keeping with the functional independence described earlier, neural circuits responsible for bird-song (procedural memory-like) and spatial memory (episodic memory-like) appear distinct in songbirds.

It is possible that the HP lesions in the present study were not large enough to affect the learning of song or responses to it. This idea seems unlikely, however, as they were sufficient to substantially compromise a “traditional” HP-dependent function. Specifically, in the same set of birds, HP lesions both in adulthood and during development caused a decrease in spatial memory acquisition as adults. Our findings are in excellent agreement with the documented decrement in spatial memory performance following compromise of HP circuits in chickadees (Sherry and Vaccarino, 1989), dark-eyed juncos (Hampton and Shettleworth, 1996) and zebra finches (Patel et al., 1997a; Watanabe and Bischof, 2004), as well as a variety of mammalian species (Squire, 2004).

Potential Roles of Song-Induced IEG Expression in the HP

Given that neurons in the HP respond selectively to conspecific songs (Bailey et al., 2002; Bailey and Wade, 2003, 2005), the question remains as to what the function of this IEG response is. The HP may modulate the activity of other regions to apportion proper responses to specific song stimuli, based on cells within the region encoding information about the environment in which relevant auditory signals are heard. Evidence for this potential function of the HP comes from male zebra finches that underwent their first courtship of a female: FOS immunoreactive neurons were observed throughout the HP (Sadananda and Bischof, 2002), expression that was higher

than that seen in males from an aviary with social and sexual contact with conspecifics. Again, this activity within the structure broadens the role of the HP over and above spatial memory processing. IEG activity in the HP following acquisition in a spatial memory paradigm (see Herdegen and 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 (Zhang et al., 2002), suggesting no involvement of the IEG in this type of memory consolidation.

IEG expression in many regions of the avian brain, the HP included, is modulated by social, auditory, and general environmental context. 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 HP than that in birds transported to the familiar site and that did not home (Shimizu et al., 2004). Adult songbirds show little to no IEG expression in Area X following song exposure (Jarvis and Nottebohm, 1997; Jin and Clayton, 1997; Jarvis et al., 1998) 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 et al., 2005), suggesting that social context modulates IEG activity within this auditory region. Given the well-defined role of the HP in memory, coupled with song-induced IEG expression in cells within the structure, HP-mediated modulation of future behavior to song stimuli remains an intriguing possibility.

CONCLUSIONS

The current experiment was essential to determining the involvement of the songbird HP in song-related and nonsong-related behaviors, and the data clearly point to a dissociation between the memory systems subserving them. Although the region does not appear to be important for song learning or production, the song-specific responses of it determined previously and its role in spatial memory are suggestive of a modulatory role for the structure in vocal communication in zebra finches, acting with other brain regions to gauge the relevance of particular stimuli, appropriately linking them with some behavioral response.

We thank Casey Bartrem (MSU) for help with histology, Christopher Chen (Lehigh) for behavior testing, and members of the Wade and Saldanha labs for care of the birds.

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