Neural Basis of Song Perception in Songbirds

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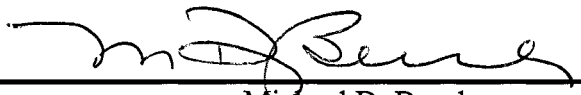

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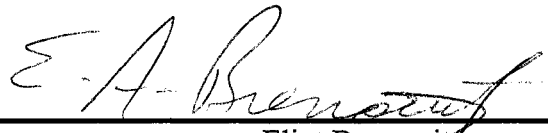

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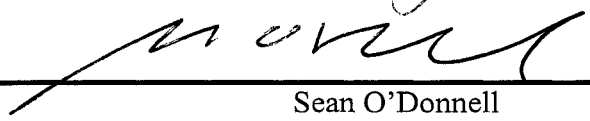

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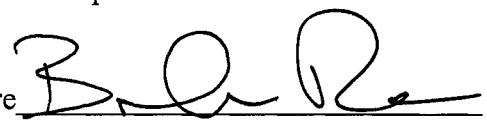
Signature 

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Abstract

Neural Basis of Song Perception in Songbirds

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Brain nuclei of the song control system involved in song perception change in size between seasons. It has been hypothesized that seasonal regression of song nuclei may impair song discrimination (Cynx and Nottebohm, 1992). We predicted that song sparrows (*Melospiza melodia*) with regressed song systems would have greater difficulty in discriminating between similar songs. Birds did not differ in their ability to learn to discriminate between shared song types.

Phosphorylation of cAMP response element binding protein (pCREB) plays a role in memory formation. Playback of conspecific song to zebra finches (*Taeniopygia guttata*) induced pCREB in HVC (Sakaguchi 1999). We played recordings of either the novel or familiar songs to wild male song sparrows. HVC of birds exposed to stranger song showed higher levels of pCREB relative to surrounding tissue. This suggests that pCREB levels in HVC may be related to learning song memories in birds

Female song recognition provides an opportunity to investigate the role of the song control system in recognition. We attempted to map auditory responses based on the phosphorylation of CREB in HVC and CMM in response to novel or familiar song. The females did not respond to the playback and likely did not

form long term memories of the song as pCREB levels remained at basal levels. pCREB increased in two birds but it was unclear whether the increase was related to their behavioral response or experience with the song.

The anterior forebrain pathway (AFP) is involved in song learning and perception. Lesions to the AFP reduce a songbird's ability to make discriminations in operant conditioning tasks (Scharff et al., 1998; Burt et al., 2000). We investigated whether the AFP plays a role in wild male song sparrows that must discriminate neighbor and stranger songs for successful territory defense. We predicted that lesions to the AFP in would impair bird's ability to discriminate songs. Birds did not discriminate between songs following lesions to the anteromedial forebrain. The inaccuracy of the lesion sites makes the result difficult to interpret. The lesions may have damaged axons projecting from HVC to Area X.

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I thank the graduate and post-doctoral students of the bird labs that taught me how to be a scientist: John Burt, J. Cully Nordby, Chris Hill, Adrian O'Loghlen, Liz Campbell, Chris Templeton, Kira Wennstrom, Tony Tramontin, Tim Billo, Chris Thompson, Anne Marie Wissman, and Adam Smith. And I thank Karin Lent for teaching me neurobiological techniques with an endless supply of patience.

Most of all, I wish to thank my fiancée Alison Leary for being my biggest supporter, as well as statistical consultant, manuscript editor, and presentation audience.

DEDICATION

To Dad

CHAPTER I: Seasonal changes in avian song control circuits do not cause seasonal changes in song discrimination in song sparrows

INTRODUCTION

Song production in oscine passerines (songbirds) is a learned behavior that plays an important role in territory defense and mate attraction (Catchpole and Slater, 1995). A discrete network of neural circuits controls song production and learning. The motor pathway for song production consists of projections from the thalamic nucleus uvaeformis (Uva) and the nucleus interface of the nidopallium (NIf) to the nidopallial nucleus HVC. HVC projects to the robust nucleus of the arcopallium (RA) in the forebrain and RA projects to the tracheosyringeal portion of the hypoglossal nucleus in the brain stem (nXIIts). Motor neurons in nXIIts innervate the muscles of the syrinx, the avian vocal organ, as well as other brainstem nuclei that innervate muscles that control respiration (reviewed by Bottjer and Johnson, 1997; Margoliash, 1997). The anterior forebrain pathway (AFP) is necessary for song learning and perception (Bottjer et al., 1984; Doupe and Solis, 1997; Margoliash, 1997; Brainard and Doupe, 2000). This pathway consists of projections from HVC to the basal ganglia homologue, Area X, from Area X to the dorsolateral nucleus of the medial thalamus (DLM), from DLM to the lateral magnocellular nucleus of the anterior nidopallium (lMAN), which projects to RA, and also back to Area X (Okuhata and Saito, 1987; Nixdorf-Bergweiler et al., 1995; Vates and Nottebohm, 1995).

These song circuits receive auditory input and neurons in both circuits show selective responses to conspecific song in anaesthetized birds (Margoliash,

1983; Doupe and Konishi, 1991). Inactivation of HVC, Area X or lMAN produces deficits in song discrimination (Brenowitz, 1991; Del Negro et al., 1998; Scharff et al., 1998; Burt et al., 2000). These observations show that song nuclei play a role in the perception of song.

In seasonally breeding songbirds, there are pronounced seasonal changes in the volume and neuronal attributes of HVC, RA, Area X, and nXIIts (reviewed in Tramontin and Brenowitz, 2000). These morphometric changes are primarily regulated by the changes in circulating plasma testosterone (T) in response to changes in photoperiod. Given the role of song nuclei in song perception, one can hypothesize that the seasonal changes in these regions may cause seasonal changes in perception (Alvarez-Buylla et al., 1990).

Cynx and Nottebohm (1992) described a photoperiodic effect on song discrimination in the zebra finch (*Taeniopygia guttata*). Zebra finches maintained on long days (LD) required fewer trials to learn to discriminate between own song, familiar song, and novel conspecific songs compared to short day (SD) birds. This apparent seasonal change in discrimination ability is somewhat surprising because breeding in zebra finches is thought to be primarily stimulated opportunistically by unpredictable patterns of rainfall rather than being synchronized with predictable changes in photoperiod (Hahn et al., 1998). Furthermore, manipulations of circulating plasma T, such as castration, do not produce changes in the size of song nuclei in adult male zebra finches (Arnold, 1980). Because of these factors, interpreting the Cynx and Nottebohm results with reference to the hypothesis of seasonal change in song discrimination is not

straightforward. As a more direct test of the hypothesis, we compared the performance of male song sparrows (*Melospiza melodia*) with fully-grown or regressed song control systems on operant conditioning tasks discriminating conspecific songs.

Song sparrows are highly seasonal breeders and show large seasonal changes in morphometric attributes of the song nuclei as well as in song structure (Smith et al., 1997; Tramontin and Brenowitz, 1999). Song sparrows have medium sized repertoires of complex songs that they use during territory establishment and defense. Song sparrow singing interactions are characterized by song-type matching. In western North American populations, neighbors share (i.e. have very similar) song types and a bird will preferentially use the song types he shares with a particular neighbor when communicating with that neighbor (Beecher et al., 1996; Beecher et al., 2000; Burt et al., 2001). We used shared song types recorded from a wild population to measure discrimination abilities in LD+T and SD song sparrows using a GO/NO-GO operant conditioning procedure. We tested the hypothesis postulated by Cynx and Nottebohm (1992) that SD birds, with their regressed song systems, would have more difficulty discriminating and require more trials to learn complex discriminations than LD+T birds with fully grown song systems. We progressively increased the degree of song similarity to determine at what point SD birds would have more difficulty than LD+T birds discriminating between shared song types. The LD+T and SD groups in our study, however, did not differ in their ability to learn to discriminate between shared song types.

METHODS

Subjects, collection and housing. We collected song sparrows at the Skagit Wildlife Reserve in Conway, WA and at Discovery Park in Seattle, WA. Birds were mist-netted after completion of the autumnal feather molt between Oct and Dec in both 1998 and 1999. All birds were sexed by laporotomy and housed in outdoor aviaries. Skull pneumatization was observed in order to verify that birds were adults (Pyle, 1997). Each year, birds were moved to an indoor aviary and housed on 8L: 16D for two to four months (depending on capture date) until all birds were photosensitive. Food and water were available ad libitum until the experiments began. Upon achieving photosensitivity, half of the birds were photoshifted one hour per day to a daylength (16L: 8D) typical of the breeding season. These birds were kept on LD for the remainder of the testing. Evidence from a closely related species, the white-crowned sparrow (*Zonotrichia leucophrys*), shows that when transferred to long days overnight the HVC grows to breeding season levels within seven days, and RA and Area X grow fully within 21 days (Tramontin and Brenowitz, 2000). Furthermore, laboratory studies have shown that increased circulating T levels, nuclei size, and song rate are influenced by social cues (Moore, 1982; Tramontin and Brenowitz, 1999). For that reason, the long day group was housed in the same room (but separate cages) with female song sparrows to provide cues that would not only stimulate the growth of the song nuclei, but also enhance their behavioral reproductive state.

Circulating T levels measured after 4 weeks on LD alone did not reach wild breeding levels in all birds (Wingfield and Hahn, 1994); this failure is typical when wild birds are brought into captivity (Smith et al., 1997). We therefore gave each LD bird a Silastic implant (I.D. = 0.762mm, O.D. = 1.8mm, length = 25 mm) containing sufficient T to raise plasma levels to the range typical of wild breeding song sparrows (Wingfield, 1984), and to ensure full growth of the song nuclei (Smith et al., 1997). The behavioral testing of the LD+T group began three weeks after T was implanted to ensure that the song control nuclei had fully grown. The SD group remained on the same 8L:16D schedule for the three month duration of the experiment. We used three LD+T and five SD birds in experiment 1 (1998) and six LD+T and four SD birds in experiment 2 (1999).

At the onset of training, birds were food restricted to 85-90% of normal feeding weight. This weight reduction is necessary to motivate the birds to perform the operant conditioning task. Water was available at all times.

Experimental test and design. A GO/NO-GO procedure was used to evaluate the discrimination abilities of the song sparrows. The operant chamber was located in a sound isolation chamber. The bird sat on a perch and was able to operate two adjacent lighted keys in a Plexiglas panel. The speaker that played all stimuli was located approximately 0.5 meters behind the subject. A PC computer controlled the behavioral program, house and panel key lights, food hopper, and recorded all key pecks made by the bird. The software to test and train the bird in the operant setup was custom written (J. Burt).

The trial began when the bird pecked the left, or "observing key", and an auditory stimulus was played. Sound files were adjusted to play at peak amplitude of approximately 65 dB SPL. A song was arbitrarily placed in either the GO or NO-GO category. A correct response to a GO stimulus was to peck the right, or "response key", which was reinforced by the extension of the food hopper for 2.5 sec. A correct response to the NO-GO stimulus was to refrain from key pecking and wait 3 sec for the trial to end. An incorrect response to the NO-GO stimulus, a peck, resulted in the house light being extinguished for 8 sec. An incorrect response to a GO stimulus (failure to key peck) had no punishment or reward. The computer randomly selected a GO stimulus 40% of the time and a NO-GO stimulus the remaining 60% of the time. A smaller percentage of GO stimuli was necessary to deter the birds from adopting an "always peck" strategy. In addition, a correction trial (i.e., a repetition of the previous trial) was presented after incorrect pecks to a NO-GO stimulus until the bird responded correctly. The bird's weight was monitored with a scale attached to the operant testing setup, and in the event that a bird did not work during an operant testing session supplemental food was provided to maintain bird's health.

In experiment 1, birds were initially trained to discriminate between a 5 second pure tone (1 kHz) and white noise in order to become familiar with the operant conditioning setup. Birds were then presented sequentially with four pairs of songs. When a bird had learned to discriminate one pair, it was presented with the next pair. The first discrimination was between two unshared song types from two singers. Succeeding song pairs were progressively more similar and

thus more difficult to discriminate (Figure 1.1A). Our assumption that more similar songs (as we define them) are indeed more difficult for birds to discriminate has been confirmed by previous perceptual experiments with song sparrows in our laboratory, as well as companion field studies (Beecher and Stoddard, 1990; Stoddard et al., 1992; Horning et al., 1993; Beecher et al., 1994). The second pair of songs represented a low degree of sharing, with approximately 50% of the notes shared between the two songs, and the third pair was more similar yet. The fourth song discrimination was between songs that shared all note types. A unique stimulus set was made for each bird in each treatment group to avoid pseudoreplication (Kroodsma, 1986).

In experiment 2, once the bird was trained to use the operant conditioning apparatus by discriminating the pure tone vs. white noise, we wished to make the song discrimination task even more difficult. We dropped the second and third levels of similarity while keeping the fourth and adding a fifth, nearly identical pair of songs (Figure 1.1B). Indicating the level of similarity by Roman numerals, we used levels I-IV in experiment 1 and levels I, IV, and V in experiment 2. As in experiment 1, a unique stimulus set was created for each bird and counterbalanced across the other treatment group to avoid pseudoreplication.

Two SD birds from experiment 2 were dropped from the experiment because we were unable to train them to use the operant conditioning apparatus. Neither of the birds was presented an auditory stimulus and thus was not included in any of the behavioral, morphological, or hormonal data or statistical comparisons.

The discrimination was considered learned when a bird responded correctly at a rate of 80% or higher for six non-consecutive 50-trial sessions. The non-breeding decrease in volume of song control nuclei could also affect the song sparrow's ability to perform the perceptual task by reducing their memory recall of song types. To determine if a difference in memory retention existed between the SD and LD+T groups, after the bird learned to discriminate all four pairs of songs, all stimulus pairs were presented within the same testing session in randomized order. The computer determined the presentation of stimulus songs randomly. Each song was presented to the bird a total of 50 times.

Songs were recorded at Discovery Park in Seattle, WA and digitized using the software program Syrinx (J. Burt). Stimuli were selected from nearly 15 years worth of recordings and represented the most similar and presumably difficult conspecific song pairs that song sparrows would have to discriminate when defending a territory. Discriminating between shared songs is a challenging and ecologically relevant task that territorial male song sparrows routinely make between territory neighbors (Beecher and Stoddard, 1990; Stoddard et al., 1992; Beecher et al., 1996; Beecher et al., 2000).

Hormone assay. Blood samples were taken every two to three weeks during the study to measure the circulating plasma T levels of birds in each group. We collected 400 µl of blood by alar venepuncture into heparinized Microtainer plasma separator tubes (Becton and Dickinson, Franklin Lakes, New Jersey). The blood was immediately centrifuged and the plasma was removed and stored at –70°C until assay. Plasma T for all birds was measured in a single

radioimmunoasssay using the Coat-A-Count Total Testosterone kit (Diagnostic Products, Los Angeles, CA). The use of this assay to measure plasma T has been validated for birds (Tramontin et al., 2001). The intra assay variability was 5% CV. The minimal detectable plasma T concentration was 0.1 ng/ml. Samples with undetectable levels of T were treated as having concentrations at this detection limit.

Brain histology and morphometry. At the completion of behavioral testing, birds (from experiment 2 only) were deeply anesthetized by methoxyflurane inhalation and perfused through the heart with heparinized saline followed by 4% paraformaldehyde. Brains were post-fixed for 24 hours in 4% paraformaldehyde at 4°C. Brains were embedded in gelatin and immersed for 24 hours in 20% sucrose: 10% neutral buffered formalin at 4°C. They were frozen on dry ice and stored at -70° C until sectioning. We sectioned brains at 50 μm on a freezing microtome and stored sections at 4° C in 0.75% saline. Alternate sections were stained with thionin. Each section containing either HVC, RA, Area X, or lMAN from both hemispheres was projected onto a piece of paper (46X) and the Nissl defined borders of each were traced in order to calculate the volume of each nucleus. We scanned the tracings into a microcomputer and measured its Area using NIH image 1.57 (Wayne Rasband, National Institutes of Health, Bethesda, MD). The volume of each section was calculated using the formula for a cone frustum (Smith et al., 1995).

Statistics. The number of trials required to reach the 80% correct criteria for each operant task were compared using two-tailed t-tests. The number of trials

required to learn to discriminate each pair was combined based on level of stimulus similarity (i.e. I-V), and compared between treatment groups. Data from the two different experiments were analyzed separately. The bird's ability to retain the discrimination was measured with a t-test comparing the percent correct performance of each bird, again pooled based on level of similarity and compared across treatment groups. Data from the two experiments were not combined for the analysis.

RESULTS

Plasma T levels. The SD birds all had undetectable levels of circulating plasma T. Birds in the LD+T group had circulating plasma T levels that equaled or exceeded those of wild birds in breeding condition (Figure 1.2) (Wingfield, 1984). Testes were visually inspected following the perfusion and all LD+T males had enlarged testes, whereas the SD group had small, regressed testes.

Behavioral results

Experiment 1. The two groups of birds did not differ significantly in their ability to learn to discriminate between the successive pairs of stimuli (Table 1; Figure 1.3). The SD birds with regressed song systems did not show any deficit in their ability to discriminate conspecific song. There was a slight trend for the birds in both groups to perform better with each sequential pair, despite the increasing similarity of the songs in successive pairs. This trend is likely the result of the birds becoming more familiar with the testing setup and the behavioral paradigm (Scharff et al., 1998). Improved performance over successive discriminations due

to increasing familiarity with the task is a well-established principle in learning experiments (Harlow, 1949). This phenomenon, often called "learning to learn", would counter the increasing similarity of the song pairs in successive discriminations.

The groups did not differ significantly in their ability to retain the previously learned discriminations (Figure 1.4). When presented with all four pairs of stimuli both groups performed comparably ($t_{(30)} = 1.031$, $p= 0.311$; $t_{(30)} = 2.768$, $p= 0.010$; $t_{(30)} = -0.181$, $p = 0.858$; $t_{(30)}= -0.356$, $p= 0.724$). The significant difference on the second retention of the pairs was likely due to an outlier; bird #502 responded correctly in only 7 out of 50 trials for one stimulus, but performed adequately on other stimuli.

Experiment 2. Despite the increased similarity of the shared song types, the two groups did not differ significantly in their ability to discriminate between the song types (Figure 1.5). Once again, the SD birds did not show any behavioral deficit (Table 1). Just as in experiment 1, both groups learned each successive task in fewer trials despite the increasing level of similarity.

The memory retention task gave results similar to those in experiment one (Figure 1.6). The SD and LD+T groups did not differ in their retention of the ability to discriminate the three pairs of stimuli based on the number of correct responses given in 50 trial presentations ($t_{(30)} = -1.266$, $p= 0.215$, $U = 290.00$, $P= .861$, $t_{(30)} = -.663$, $p= 0.512$; a Mann-Whitney U Test was performed on the data from stimulus pair 2 because they failed the equal variance test). Additionally, a repeated measures ANOVA evaluating all three retention

tasks simultaneously within individuals showed no significant differences between SD and LD+T groups (F=0.094, p=0.830)

Brain histology and morphometry. HVC, RA, and Area X were all significantly larger in the LD+T birds relative to the SD birds in experiment 2 (HVC: $t_{(9)}$ = 3.642, $p \leq .005$; RA: $t_{(9)} = 6.375$, $p \leq .001$; Area X: $t_{(7)} = 2.456$, $p \leq .044$, t test; Figure 1.7). The volume of lMAN did not change seasonally ($t_{(8)} = -1.062$, p = 0.319, t test; Figure 1.7). The volumes of the song nuclei in the two treatment groups were comparable to those seen in previous studies of this species (Smith et al., 1997; Tramontin et al., 2001).

In experiment 2, there was no correlation between the volume of HVC and the number of trials required to learn each of the three discriminations independent of seasonal condition (I: r = -.264, p = .461; IV: r = -.191, p = .598; V: r = .022, p = .956). There were, however, weak trends suggesting that the volume of lMAN may be related to performance on the behavioral tasks; greater lMAN volume was associated with fewer trials to learn each discrimination (I: r = -.473, p= .199; IV: r = -.333, p= .381; V: r = -.632, p =.093), and also with percent correct on the retention task (I: r = .600, p= .116; IV: r = .703, p= .052; V: r = .525, p =.182).

DISCUSSION

Song sparrows with regressed song nuclei were as adept at learning complex song discriminations in an operant paradigm as birds with fully-grown song nuclei. Volumes of HVC, RA, and Area X differed between the two groups

but there were no differences in song discrimination. These data suggest that seasonal differences in song discrimination do not occur in this species.

Previous research described a seasonal difference in song perception in another songbird, the zebra finch (Cynx and Nottebohm, 1992). Those investigators found that LD birds learned to discriminate between a single pair of conspecific songs in fewer trials than SD birds. Our study differed from that of Cynx and Nottebohm (1992) because we attempted to mimic the key features of the perceptual task required of a song sparrow defending his territory, rather than the bird's ability to discriminate familiar songs. Song sparrows must discriminate between neighbors with similar songs and determine whether the neighbors are singing from the proper territory boundary. A neighbor singing from a new location (e.g. opposite boundary) is considered just as threatening as a singing stranger (Stoddard et al., 1991). Our stimulus set represented the range of song similarity found in the song repertoires of neighboring song sparrows in our population, and included extreme examples of song similarity and thus the most difficult discriminations a bird would need to make in nature.

Cynx and Nottebohm (1992) first described the hypothesis based on data from the opportunistic-breeding zebra finch. T levels and brain nuclei were not measured to verify that the zebra finches had in fact responded physiologically to the change in daylength, or that any changes were specific to the song control system. The song sparrows used in our study exhibited pronounced seasonal changes in brain morphometry and circulating plasma T levels.

Operant conditioning was used both in our and the Cynx and Nottebohm study, so the different result is probably not attributable to the behavioral assay. Also, both studies used the number of trials to reach criterion as a measure of the birds learning the task. One possible reason for the superior performance of LD birds in the Cynx and Nottebohm study on a similar behavioral task is an overall enhancement of activity on long days. Cynx and Nottebohm used perch-hopping as their conditioned response. This was likely a more energetically demanding response than the key-pecking assay we used and therefore perhaps more subject to nonspecific seasonal differences in general activity level. In the present study, we found that it was harder to motivate SD birds to peck keys for food, especially in the initial training. In fact, we had to drop two SD subjects from experiment 2 because they could not be motivated to perform during the three months of behavioral testing. Moreover, SD birds often required more time to complete their 50 trial sessions than LD birds and would go several days without working, though their discrimination performance on these trials was similar. Because Cynx and Nottebohm did not have a control task (e.g. visual discrimination, Burt et al., 2000), it is impossible in their study to distinguish general from specific effects of the day length manipulation.

It is possible that we did not detect a seasonal difference in song discrimination because song sparrows defend their territories year round, although much less intensely outside of the breeding season (Wingfield, 1994). Therefore song sparrows may need to recognize their neighbors throughout the year and, consequently do not show a seasonal difference in song perception. This scenario

could be tested in a species that shows seasonal changes in the size of the song nuclei, but does not defend territories year round. Our data do indicate, however, that seasonal changes in song nuclei need not cause a seasonal change in song discrimination.

Another alternative explanation is that we masked a song discrimination effect of long days by implanting testosterone. Exogenous testosterone is needed to stimulate the breeding condition of a male song sparrow when in the unnatural conditions of the laboratory. Previous research has shown that there are no differences in the ability to discriminate conspecific songs between male zebra finches castrated and implanted with either T or empty silastic pellets (Cynx and Nottebohm, 1992). However, our stimulus set consisted exclusively of conspecific songs, such as a wild song sparrow would here on its territory, and thus would predict that T implants would not mask any discrimination abilities in our birds.

Our data correlating lMAN volume and the bird's performance on the behavioral tasks tentatively suggests that lMAN may play a role in maintaining song discrimination abilities across reproductive conditions. This hypothesis is consistent with the known auditory characteristics of lMAN (Doupe and Konishi, 1991; Doupe, 1997) and the results of previous lesion studies of lMAN's role in song perception (Scharff et al., 1998; Burt et al., 2000).

Our results provide insights into understanding the role of song circuits in song discrimination and the functional significance of seasonal plasticity in the brain. It is clear that seasonal changes in the ability to make challenging song

discriminations are not an inevitable consequence of pronounced morphological and physiological changes in the song control nuclei. These observations raise two possible hypotheses about the relationship between song discrimination and the song control circuits. One possibility is that song discrimination abilities may be maintained across seasons by song related brain regions, such as lMAN, that do not change seasonally. Alternatively, discrimination may be regulated by nuclei that do undergo seasonal change, but these regions may be adequate for discrimination even in their regressed non-breeding state. Seasonal plasticity of the song circuits may be more directly related to changes in the motor aspects of song production and energetic demands imposed by the song system (Tramontin and Brenowitz, 2000; Wennstrom, 2001)

TABLE 1.1. Mean number of trials to criterion and standard error for each pair of stimuli (I-V). There were no significant differences between the SD and LD+T birds on any of the stimulus pairs (t tests).

Stimulus Pairs	I		II		III		IV		V	
	mean	std err	mean	std err	mean	std err	mean	std err	mean	std err
Expt. 1 SD	1345	516.31	1060	259.58	885	179.26	760	100.80		
Expt. 1 LD+T	1329	282.50	669	118.51	1284	154.93	1070	240.29		
n=6	t=-0.022	p=0.983	t=-1.097	p=.315	t=1.506	p=0.183	t=1.40	p=0.211		
Expt. 2 SD	1959	666.01					1366	161.78	1910	450.45
Expt. 2 LD+T	1725	261.37					1503	295.10	1573	131.56
n=8	t=-0.377	p=0.716					t=0.35	p=0.735	t=-.903	p=.401

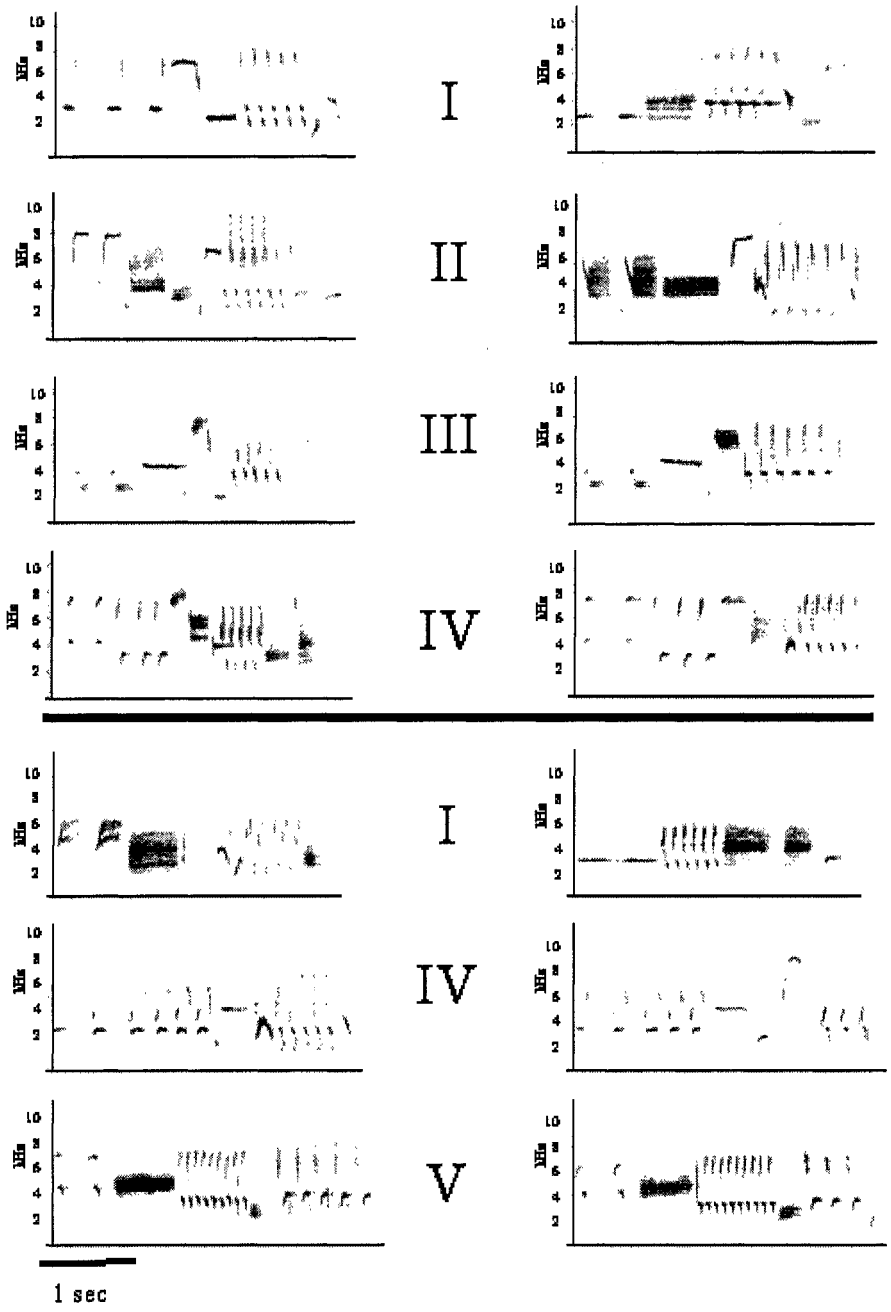

Figure 1.1. Sound spectrograms of the song types used in both experiment 1 (I-V) and experiment 2 (I,IV, and V). Comparison of song types in each row shows that the level of note sharing (i.e. similarity) increased progressively throughout both experiments.

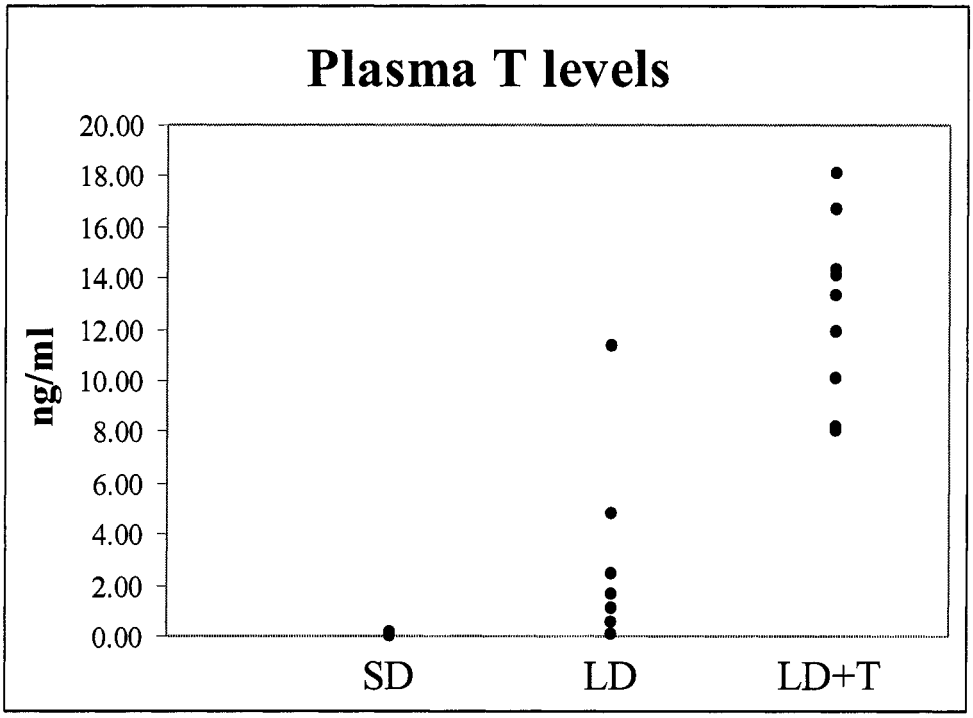

Figure 1.2. Mean circulating plasma T levels for SD birds, LD birds prior to T implant, and LD birds with T implant.

Experiment 1: Retention

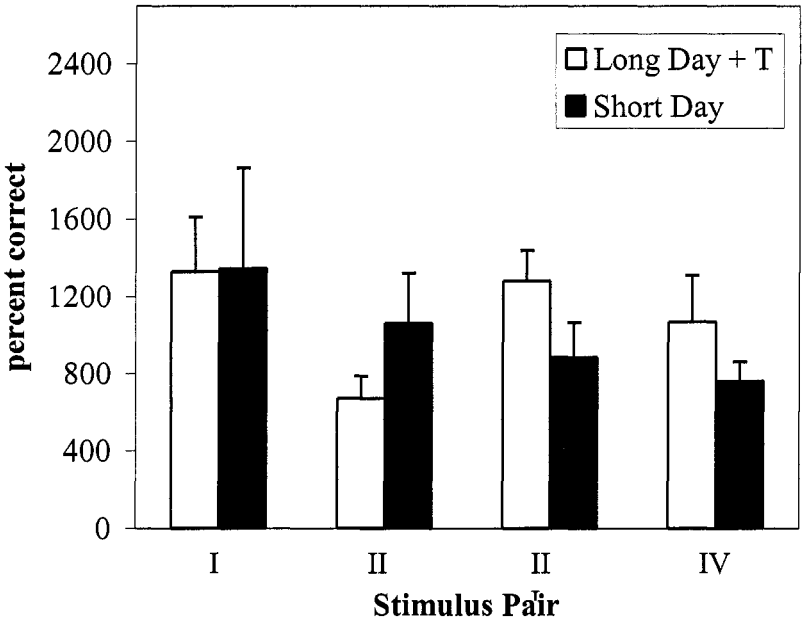

Figure 1.3. Mean (+ SE) number of trials required to reach 80% correct criterion for experiment 1.

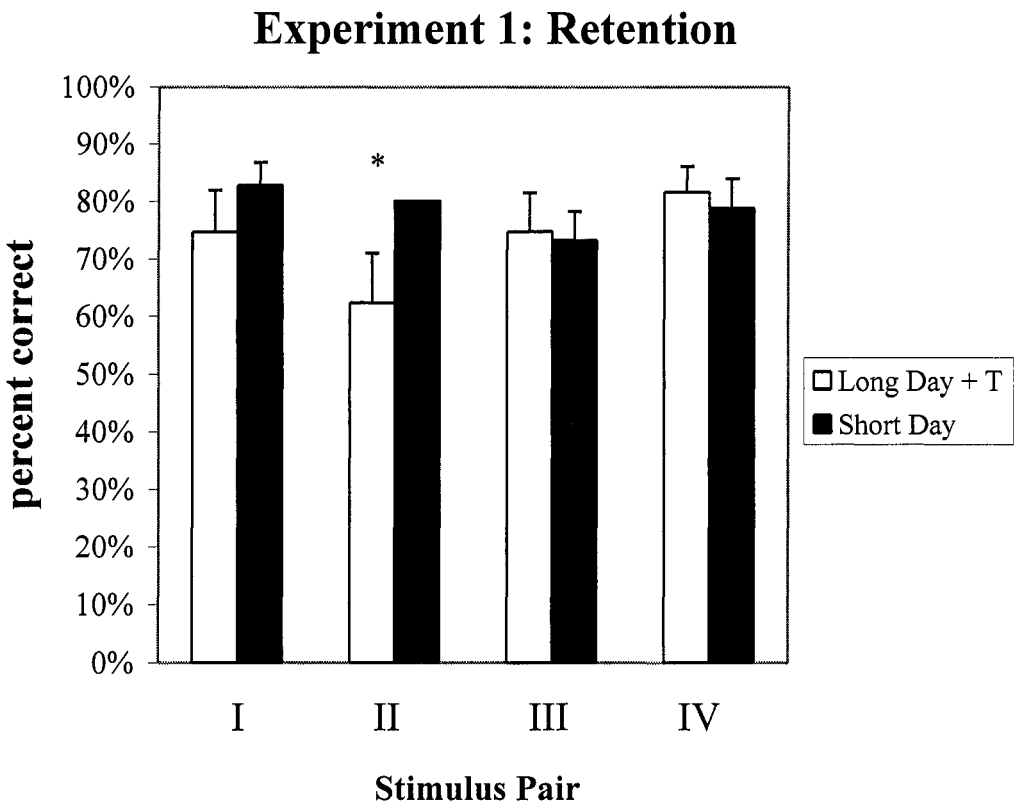

Figure 1.4. Mean (+ SE) percent correct of retention from experiment 1

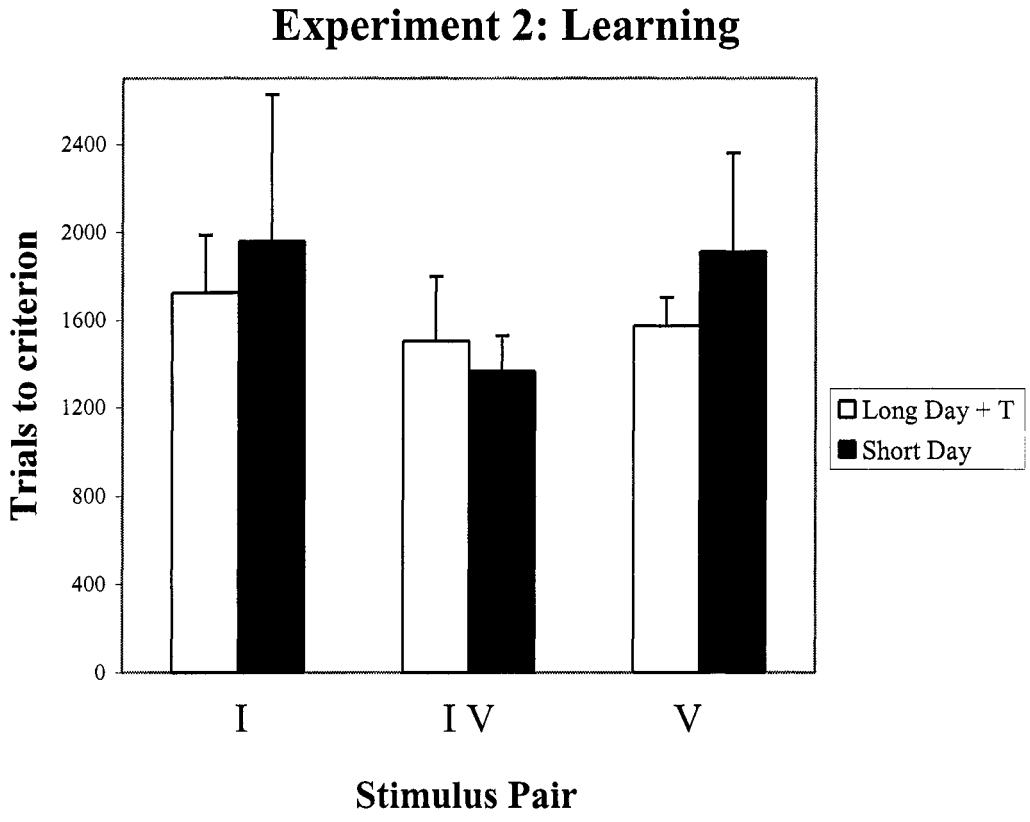

Figure 1.5. Mean (+ SE) number of trials required to reach 80% correct criterion for experiment 2.

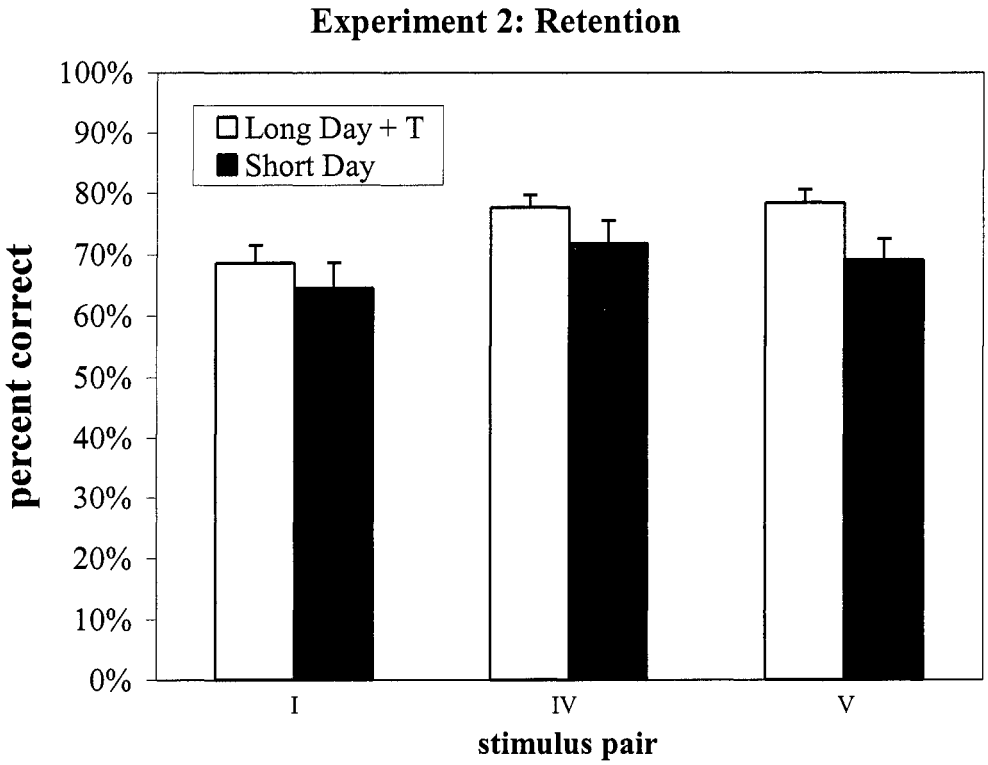

Figure 1.6. Mean (+ SE) percent correct of retention from experiment 2.

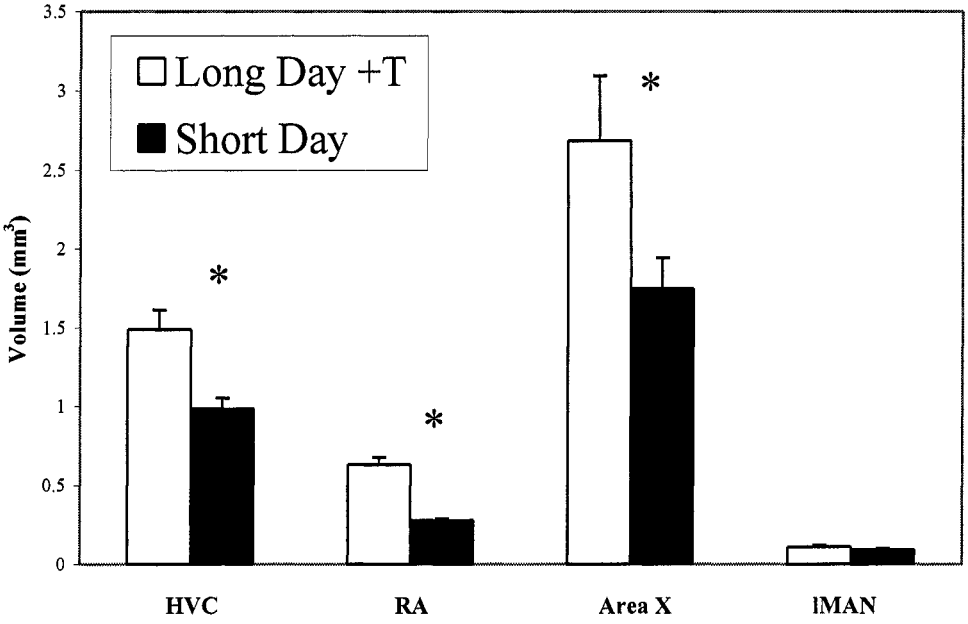

Figure 1.7. Volumes of three telencephalic song control nuclei in the SD and LD+T groups (*, $p \leq .044$, t test)

CHAPTER II: Selective CREB phosphorylation in HVC in response to presentation of novel song to wild song sparrows

INTRODUCTION

Males of most species of songbirds use song to establish and defend territories. In many songbirds, individuals sing several distinct song types to form a song repertoire. Song sparrows (*Melospiza melodia*) typically have song repertoires of 7-10 song types, and neighbors learn to recognize one another's song repertoires (Stoddard et al., 1991; Stoddard, 1996). Because recognition is based on song type and not voice characteristics (Beecher et al., 1994), territory maintenance between neighboring song sparrows requires recognition of all the song types in each neighbor's repertoire (Stoddard et al., 1991; Burt et al., 2001). Neighboring song sparrows therefore provide a natural experiment of long term memory formation with an easily monitored behavior that is controlled by a described and discrete neural network. We used this natural experimental model to investigate the neural basis of song recognition and long term memory formation in the song control system of songbirds.

The songbird song control system is a network of nuclei involved in song learning, perception, and production. Song behavior is regulated by two main circuits (Figure 2.1), the motor pathway and the anterior forebrain pathway (AFP). The nucleus HVC (used as proper name) plays a central role in both circuits. The AFP is essential for song learning during development and song maintenance in adulthood, but is not required for song production (Bottjer et al., 1984; Scharff, 1991; Williams and Mehta, 1999; Brainard and Doupe, 2001).

Phosphorylation of the transcription factor, cAMP response element binding protein (CREB), is necessary for the formation of long-term memories (Bourtchuladze et al., 1994). CREB is thought to mediate transcription processes early in long-term memory formation that stabilize synaptic changes stimulated by learning (Matynia et al., 2001). Increased CREB phosphorylation (pCREB) may also allow neuronal circuits to acquire memories more rapidly (Silva et al., 1998). Silva et al. hypothesized that, through evolution, CREB has adapted to various types of memory systems according to their computational capacity and storage ability. Increased CREB phosphorylation may be associated with learning in situations that are critical for survival and reproduction. For example, during the breeding season there could be strong selective pressure for a male songbird to rapidly learn to identify a potential territorial competitor in order to successfully mate and reproduce. The need to maintain a mating territory selects for songbirds to rapidly process and store information. Thus, CREB phosphorylation could be required to learn to recognize the individually distinct songs of a potential competitor. We carried out a field study with song sparrows to investigate whether CREB phosphorylation is involved in the formation of new or retrieval of previously learned auditory memories in the song control system.

Sakaguchi et al. (1999) reported for zebra finches that there was greater pCREB immunoreactivity in HVC neurons in response to playbacks of conspecific song than to either heterospecific song (canary) or white noise. It is difficult to interpret their result in a functional context, because these investigators did not use any behavioral assays of the birds' responses to the stimuli. HVC is

known to play a role in song perception (Brenowitz, 1991; Del Negro et al., 1998; Gentner et al., 2000), but it is unclear whether song memories are formed and/or or stored within HVC. At least two hypotheses consequently can be proposed to explain the results of Sakaguchi et al. 1) Increased CREB phosphorylation in HVC neurons may be associated with the formation of new memories of a novel and biologically relevant song; or 2) induction of pCREB in HVC in response to the presentation of conspecific song may be related to a retrieval or recognition process.

Song sparrows defend territories year round, but are especially aggressive in the spring during the breeding season. Once territory boundaries are established early in the breeding season, song sparrows learn to recognize the songs of their neighbors and generally do not respond as aggressively to the neighbors' songs as they do to the song of a stranger (Stoddard et al., 1990).

In the present experiment we used a neighbor/stranger discrimination paradigm to contrast CREB phosphorylation in response to familiar and unfamiliar songs in the song control system. The stranger songs, never heard before by the subjects, are threatening stimuli that the birds would have to learn quickly, whereas the neighbor songs are familiar, having been previously memorized. The memory formation hypothesis predicts that there will be more CREB phosphorylation in HVC neurons in response to the unfamiliar stranger song than to the familiar neighbor song. The recognition hypothesis, however, predicts that there will be more CREB phosphorylation in HVC neurons in response to the familiar neighbor song than to unfamiliar stranger song.

We also investigated the expression of the immediate early gene product ZENK (homologous to zif-268, egr-1, NGFI-A, and krox-24) in the song control system. ZENK is often used as an indicator of neuronal activation. Song production induces ZENK expression throughout the song control system (Jarvis and Nottebohm, 1997) and is context dependent in Area X in zebra finches (Jarvis et al., 1998). A male singing alone or in the presence of other males induces high levels of ZENK expression in Area X, whereas singing to conspecific females does not. Male zebra finches sing highly stereotyped songs to females but sing much more variable song when singing to males or singing solo (Zann, 1996). One hypothesis to explain the relationship between stereotypy and decreased ZENK expression is that there is a difference in arousal state between the two behavioral contexts (Mello, 2002). Mello posits that the increase in arousal and song stereotypy when singing to females reduces neuronal activity and thus ZENK expression in Area X. Song sparrows that sing in response to playback of conspecific song on their territory show a great deal of variability of ZENK expression in Area X (Jarvis et al., 1997), similar to the range of ZENK expression seen across contexts of zebra finch singing. We investigated whether the neighbor/stranger context in which song sparrows use their song leads to context dependent ZENK expression in Area X.

METHODS

Playbacks Behavioral data were collected at Charles L. Pack Experimental Forest in Eatonville, WA (46.868°N, 122.265°W). The birds' legs were color banded with a unique color combination so they could be identified throughout the course

of the experiment. Full song repertoires were recorded from each bird within the study population using a Sennheiser ME-88 directional microphone and Sony TCD-5Mcassette recorder. Recordings were digitized using the software program Syrinx (J. Burt, www.syrinxpc.com). Territory boundaries were measured prior to the experiment by observing the bird sing from perches and defend in response to playback. Playback of song was necessary to induce singing when recording full song repertoires before the experiment began. We used different song types from different birds during the song recording and experimental parts of the study.

We prepared a playback tape of each bird's repertoire. Each song type was played ten consecutive times at a ten second interval, 6 songs per minute for 30 minutes, for a total of 180 songs. The song repertoire of each bird repeated 1 to 1 ½ times over the 30 minute trial. Playback trials were performed between dawn and noon or at dusk late in the breeding season in June 2002 (n=12) and 2003 (n=10). In order to avoid interruption of the playback and the simultaneous audition of familiar and unfamiliar songs, the neighboring bird that shared the boundary from which the playback speaker was placed was captured in a mist net and kept until the playback with the focal bird was completed. If we failed to capture the neighbor, a caged live decoy was placed within the territory on the opposite side of the neighboring bird's territory. In response to an intruder inside the territory (or a caged decoy), a song sparrow will generally stop singing and advance to more aggressive posturing and full contact fighting (Wingfield, 1993; Sperry et al., 2003). Thus, a caged decoy on the neighbor's territory kept the

neighbor sufficiently occupied and silent. Once the neighbor was either caught or occupied, a mist net was set up furled inside the focal bird's territory for subsequent capture after the playback period.

Song playback tapes mimicking naturalistic social interactions consisted of full song repertoires of either neighbor or stranger song types. We recorded all songs used for the playback at Charles L. Pack Experimental Forest. We considered songs recorded at least 1 kilometer away from a focal bird's territory to be stranger songs, a range twice the distance that has been described before in song sparrows searching for food in the winter when territory defense becomes less rigorous (Nice, 1937; Arcese, 1989). The playback period lasted 30 minutes. This amount of time was shown to be optimal for immunolabelling of phosphorylated CREB (Sakaguchi et al., 1999). A speaker positioned in a uni-directional sound parabola (Sony PBR-330) was placed on the territory border facing the center of the focal bird's territory. Once the playback was started, the 30-minute playback trial began after the bird's first observable response to the taped songs. We considered the bird's flying toward the speaker or singing in the direction of the speaker as confirmation that the bird attended to the stimulus. We recorded the amount of time the focal bird spent within 10 meters of the playback speaker (Stoddard et al., 1990) and the number of songs produced by the target bird in response to the playback (Burt et al., 2001). At the end of the 30 minute playback, the tape was stopped briefly while the mistnet was unfurled and a caged live decoy was placed beside the mistnet. The playback was restarted until the bird was captured the mistnet. If a bird was not caught within 10 minutes the trial

was aborted and a playback was reattempted a few days later. In the case of an aborted stranger playback interaction, a different stranger song would be played when the trial was reattempted so that the stimulus would be novel. The playback trial was also aborted if a neighboring male was attracted by the playbacks and began to sing himself and/or interacted with the focal bird.

Immediately following the bird's capture a blood sample was taken to measure plasma testosterone (T) levels, a physiological index of the bird's level of aggression after the playback (Wingfield, 1990). We collected 400 µl of blood by alar venepuncture into heparinized Microtainer plasma separator tubes (Becton and Dickinson Company, New Jersey). The blood was stored on ice until it was returned to the laboratory. It was centrifuged and the plasma was separated and stored at –70°C until assay. Plasma T was measured in a single radioimmunoasssay using the Coat-A-Count Total Testosterone kit; the intra-assay variability for the kit is 5% CV (Diagnostic Products). The use of this assay to measure plasma T has been validated for birds (Tramontin et al., 2001).

The perfusion began in the field immediately after taking the blood sample. The birds were deeply anesthetized by methoxyflurane inhalation and perfused through the heart with heparinized 0.75% saline followed by 4% paraformaldehyde. Brains were post-fixed in 4% paraformaldehyde and stored in an ice filled cooler until returning to the laboratory the same day and refrigerated for 24 hours at 4°C. Brains were embedded in gelatin and immersed for 24 hours in 20% sucrose: 10% neutral buffered formalin at 4°C. Brains were frozen on dry ice and stored at -70° C until sectioning.

Morphometry and Immunohistochemistry We sectioned brains at 40 μm on a freezing microtome and stored sections at 4° C in 0.75% saline. Every third section was stained with thionin in order to locate the nuclei of interest. Sections containing Area X, HVC, and RA were immunolabelled for pCREB, CREB-BP, and ZENK. CREB binding protein (CBP), a co-activator for pCREB, was measured to control for a general change in of CREB activation in response to song (Bading et al., 1993; Chawla et al., 1998). CBP does not activate gene expression in the absence of pCREB and CREB phosphorylation is not altered by the by the addition of exogenous CBP (Chawla et al., 1998). This suggests that CBP is not a limiting factor in the downstream gene expression cascade regulated by pCREB. CBP has been shown to vary seasonally in songbirds (Auger et al., 2002), but CBP has not been shown to vary in direct response to a sensory stimulus.

Endogenous peroxidase activity was quenched by a 10 minute treatment with 0.3% H2O2 in PBS-TX (0.1 M PB + 0.9% NaCl + 0.2% Triton X-100 [Sigma]). We washed sections in PBS-TX and blocked them with 10% normal goat serum (NGS) in PBS-TX, and incubated them in primary antibody for pCREB (gift of Dr. Mark Montiminy, 1:2000), CREB-BP (Santa Cruz Biotechnologies, 1:5000), or ZENK (Santa Cruz Biotechnologies, 1:400) overnight. Sections were washed in PBS-TX, incubated in biotinylated anti-rabbit secondary antibody (Vector Laboratories, 1:200) for one hour, rinsed in PBS, incubated in avidin-biotin complex (ABC; PK-4000; Vector) for 45 minutes, rinsed in PBS, incubated with a biotinylated tyramide amplification reagent

(NEN) for 20 minutes, rinsed in PBS, incubated again in ABC for 15 more minutes, rinsed in PBS, and rinsed in 0.05M Tris for 5 minutes. The color reaction was performed with 3,3' diaminobenzidine (DAB) and allowed to develop 5-20 minutes. Sections were washed in 0.05M Tris, mounted onto gelatin-coated slides in distilled water and air-dried overnight, after which they were dehydrated in an ethanol series, cleared in xylene, and coverslipped.

Every third section through the brain was stained with thionin. Each section containing either HVC, RA, or Area X from both hemispheres was projected onto a piece of paper (46X) and the Nissl defined borders of each were traced in order to calculate the volume of each nucleus. We scanned the tracings into a microcomputer and measured its Area using NIH image 2.08 (Wayne Rasband, National Institutes of Health, Bethesda, MD). The volume of each section was calculated using the formula for a cone frustum (Smith et al., 1995).

Many of the brains immunolabelled for pCREB failed to show distinct cellular resolution. Therefore, optical densitometry (OD) was used to quantify pCREB. The amount of color product formed by the DAB color reaction indicates the amount of pCREB in the tissue. Images were captured using NIH Image (v.2.08) throughout HVC and RA while maintaining a constant light level. Area X was not visible in sections immunolabelled for pCREB and was not measured. The optical density of the images was calibrated by a 17 step rodbard standard tablet (NIH, www.nih.gov). The nucleus and surrounding tissue were traced and measured for mean optical density. A ratio of the region of interest to the surrounding tissue was then calculated. The mean density ratio for each

section was averaged within each bird to come up with a single mean density average for both HVC and RA relative to the surrounding tissue. Images of HVC and RA were captured on different days at different light intensities, thus their absolute values could not be compared. We did compare relative values of the ratios of HVC to nidopallium versus RA to arcopallium.

ZENK and CBP labeled sections showed distinct cellular resolution, therefore we counted immunolabelled cells using a random systematic sampling scheme in HVC and Area X for ZENK, and in HVC and RA for CBP. The region of interest was magnified at 4x and captured through a video camera mounted onto the microscope using NIH image. Sampling boxes were randomly created within the region of interest and magnified to 40x and an image was captured. Boxes from every section were counted to get a uniform sample across the entire nucleus, except in cases of high immunolabelling which only required samples from every other section. In cases of low immunolabelling, multiple boxes were created within the region of interest. Images of each box were overlaid with a grid (0.086 x 0.057 mm^2). All cells with a clear nucleolus were counted as neurons. Boxes were sampled until at least 150 cell profiles were counted. This sample size provides an accurate estimate of cell density equivalent to that produced by the stereological optical dissector method (Tramontin et al., 1998). For two birds we were not able to sample 150 ZENK-labeled cells because labeled cell density was so low. In order to estimate the number of cells immunolabelled for CBP and ZENK, cell density was multiplied by the Nissl defined volume of each nuclei.

RESULTS

Song sparrows that heard novel-stranger song had higher CREB phosphorylation in HVC relative to surrounding nidopallium than birds that heard familiar-neighbor song ($t_{(7)} = 3.66$, $p \leq 0.008$, Figure 2.2). There was no overlap in the pCREB OD between the two playback groups (stranger: M = 1.23, SEM = 0.01; neighbor: M = 1.14, SEM = 0.02). OD of HVC alone was compared using an independent samples t-test and there was no difference between neighbor and stranger song playback ($t_{(7)} = -3.80$, $p = 0.715$). There was also no difference in OD in the area of nidopallium just ventral to HVC that was used to calculate the HVC/nidopallium ratio ($t_{(7)} = -0.971$, $p = 0.364$). The number of songs produced of time spent within 10 m of the playback speaker did not correlate with pCREB OD in HVC ($r = 0.07$, $p = 0.93$; $r = 0.25$, $p = 0.63$).

There was no difference in OD in RA relative to surrounding arcopallium between the two playback groups ($t_{(7)} = -0.646$, $p \leq 0.54$). This was predicted because RA is in the motor pathway and is unlikely to be involved in song perception (Figure 2.3). The images of HVC and RA used to measure OD were taken on separate days and thus the absolute OD values could not be compared. There was no correlation between the ratios of HVC and RA to surrounding tissue ($r = .004$, $p = 0.992$).

A one-way ANOVA was used to compare the number of cells immunolabelled for CBP in HVC and RA. No difference was found between the two playback groups in HVC ($F_{(6)} = -1.617$, $p = 0.157$, Table 2) or RA ($F_{(5)} = -0.236$, $p = 0.823$, Table 2, Figure 2.4). The number of songs produced ($r = -$

0.090, p = 0.848) and time spent within 10 m of the playback speaker (r = 0.132, p = 0.832) did not correlate with the number of CBP immunolabelled cells in HVC.

A one-way ANOVA was used to compare ZENK expression in HVC and Area X. No difference was found between the two playback groups in HVC ($F_{(14)}$ = 0.395, p = 0.54) or in Area X ($F_{(14)}$ = 2.68, p = 0.124, Table 2, Figure 2.5). The number of songs produced (r = -0.091, p = 0.802) and time spent within 10 m of the playback speaker (r = 0.435, p = 0.158) did not correlate with ZENK expression.

There was no difference in the amount of time the subject spent within 10 m of the playback speaker between neighbor or stranger playback groups ($F_{(16)}$ = 1.507, p = 0.239, Figure 2.6). Nor in the number of songs produced ($F_{(20)}$ = 0.088, p= 0.770, Figure 2.7). We compared plasma T levels between the two groups using an independent sample t-test ($t_{(19)}$ = 1.62, p = 0.12). There was no difference in plasma T levels between the two groups (Figure 2.8). Independent sample t-tests were used to compare plasma T levels between the two groups in birds that were analyzed for either pCREB or ZENK (or both). No difference was found between the two playback groups in birds that were analyzed for pCREB ($t_{(8)}$ = -0.13, p = 0.90) or ZENK ($t_{(14)}$ = -0.112, p = 0.91).

DISCUSSION

The increase in CREB phosphorylation in HVC in response to novel and biologically relevant song playback suggests that song sparrows may have been

forming long term memories of stranger songs when defending their territory. The difference in pCREB immunolabelling of HVC is unlikely due to the motor production of song because the two groups sang an equal number of songs in response to the playback, and the number of songs produced did not correlate with CREB phosphorylation in HVC. The song rate data are also consistent with other neighbor/stranger discrimination experiments in which the number of songs produced in response to a playback is a poor predictor of discrimination (Burt et al., 2001). Also, there was no difference in CREB phosphorylation between the two groups in RA relative to surrounding arcopallium. RA is part of the motor pathway and is likely not involved in long term memory formation of another bird's song. Additionally, there was no difference in the plasma T levels between the groups, which suggests that the difference observed in pCREB OD in HVC is the result of learning and not simply a difference in CREB phosphorylation due to a difference in aggressive response to song.

The most important difference between the two groups, with regard to CREB phosphorylation, is the subject's prior experience with the playback. The subjects heard neighbors sing hundreds of songs a day during the breeding season for three months prior to the experiment. The song sparrows at Pack Forest are a non-migratory population and also heard neighbors sing throughout the year. Song sparrows defend their territories less intensely outside of the breeding season and may only hear on the order of dozens of songs from a neighbor in a day. Song sparrows undoubtedly had enough time to learn to recognize the species specific signal for which they have an extensive memory capacity

(Stoddard et al., 1992). Also, song type sharing within the population shows that neighbors were tutors during the first year of life for juveniles learning to sing. While we can not attribute the direction of learning-tutoring from one neighbor to another, it is clear the birds have been familiar with their neighbor song since their first year of life (Nordby et al., 1999).

The lack of variation in CBP between the two playback groups is an important control for CREB phosphorylation. CBP is a co-activator for pCREB that cannot activate gene transcription on its own. The lack of variation between the two playback groups strengthens the suggestion that the increase in pCREB in HVC was the result of long term memory formation of an auditory communication signal. Consistent with previous research investigating the role of CBP in neural function, CBP did not increase in a short time course in response to sensory stimulation (Auger et al., 2002; Fiore and Gannon, 2003).

The similarity in ZENK expression in HVC between the two groups further suggests that CREB phosphorylation is specifically related to memorization of novel song. As an indicator of neuronal activation, ZENK expression in HVC shows that the increase in pCREB in response to stranger song was not simply the result of more neuronal activity. In a similar playback experiment with wild song sparrows, Jarvis et al. (1997) described extensive variation in ZENK expression in Area X in song sparrows that were exposed to stranger song. We also observed similar variation in ZENK expression within Area X, but the variation was not correlated with any of our behavioral measures. There was a trend in the direction of less ZENK expression in Area X in response

to novel-stranger song than to familiar-neighbor song, but the differences were not significant. ZENK expression in Area X in zebra finches has been shown to be dependent on whether or not a male sings to a female (Jarvis and Nottebohm, 1997). The context dependent expression is thought to result from behavioral differences in arousal state (Mello, 2002). We did not observe a difference in behavioral or hormonal measures between the two playback groups. One cannot draw a direct analogy between female directed song in a zebra finch and a song sparrow defending his territory against a neighbor or stranger. A more accurate test of the relationship of arousal state and context dependent ZENK expression in song sparrow would compare song sparrows posting along the border of their territory with song sparrows defending their territory against a specific intruder.

There was no difference between playback groups in the amount of time the subject spent within 10 m of the playback speaker or in the number of songs produced between the two playback groups. At least two factors may have contributed to the similarity in behavioral response between the two groups. The playback lasted 30 minutes which is optimal for ZENK protein synthesis (Mello and Clayton, 1995), which is 27 minutes longer than in previous song sparrow neighbor/stranger discrimination tests (Stoddard et al., 1990; Burt et al., 2001). Data were not collected within time bins so we could not separately analyze data collected in the first 3 minutes, so that even if there was a differential response early in the playbacks this could have been obscured over the 30 min trial. Also, vocal interactions between familiar neighbors are generally brief, playing neighbor song for 30 continuous minutes as did may therefore have stimulated the

subject to respond more aggressively to his neighbor's song that he would normally

We found that CREB phosphorylation increases in HVC in response to playback of novel song. Our findings suggest that HVC is either the location or part of a circuit that learns the songs of conspecific males. We also describe a powerful experimental protocol to investigate the neural basis of memory formation in a free living songbird in its natural environment. Further investigations of CREB phosphorylation in response to conspecific song are needed to determine the function of auditory forebrain regions in song processing before the signal reaches HVC and what function the adult AFP may have in conspecific song recognition.

Table 2.1: Summary of the number of cells immunolabelled for CBP and ZENK protein in the oscine song control system. There were no significant differences in the number of cells labeled for CBP or ZENK in response to novel or familiar playback.

	CBP			
	HVC		RA	
	n=4 stranger	n=3 neighbor	n=4 stranger	n=3 neighbor
mean cell number	162572	123534	34877	32735
SEM	15609	16736	6522	5681

	ZENK			
	HVC		Area X	
	n=7 stranger	n=5 neighbor	n=10 stranger	n=6 neighbor
mean cell number	78152	93939	53028	93386
SEM	11545	25309	14807	20138

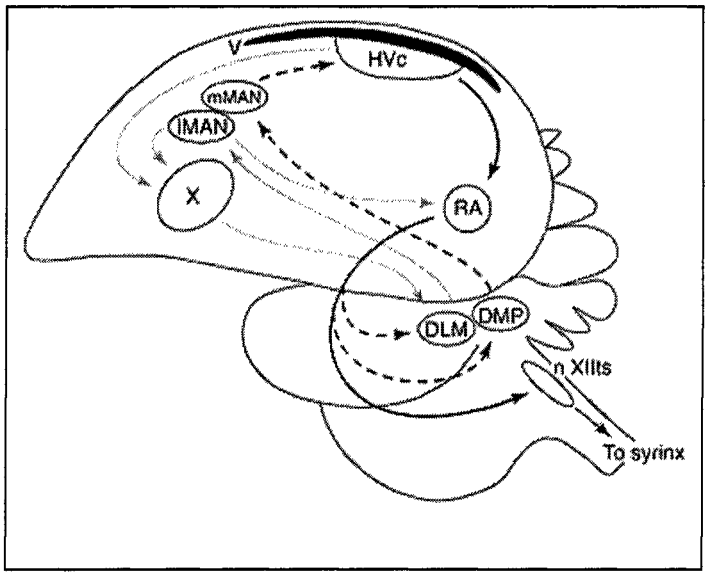

Figure 2.1: Schematic sagittal drawing of the oscine song control system. Projections of the motor pathway are shown in black and the anterior forebrain pathway in gray. The AFP consists of projections from HVC to Area X, a basal ganglia homologue, which projects to the dorsal lateral nucleus of the medial thalamus (DLM). DLM projects to the lateral portion of the magnocellular nucleus of the anterior nidopallium (lMAN). lMAN projects both to the motor pathway via the robust nucleus of the arcopallium (RA) and back to the AFP via Area X. The motor pathway for song production consists of projections from the HVC, which projects to RA, a premotor region in the forebrain. RA projects to the tracheosyringeal portion of the hypoglossal nucleus in the brain stem (nXIIts). Motor neurons in nXIIts innervate the muscles of the syrinx, the avian vocal organ, and brainstem nuclei that innervate muscles that regulate respiration (reviewed by Bottjer and Johnson, 1997; Margoliash, 1997).

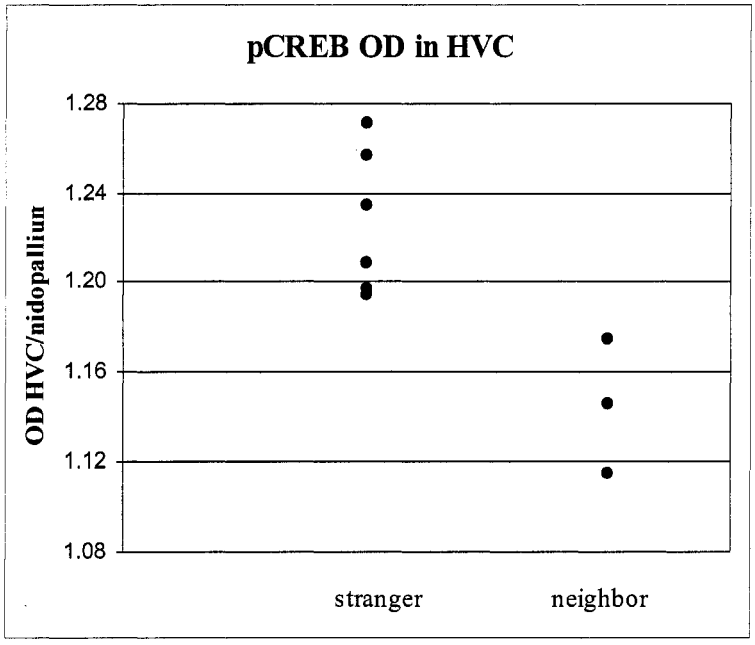

Figure 2.2: Individual pCREB OD ratios of HVC/nidopallium.

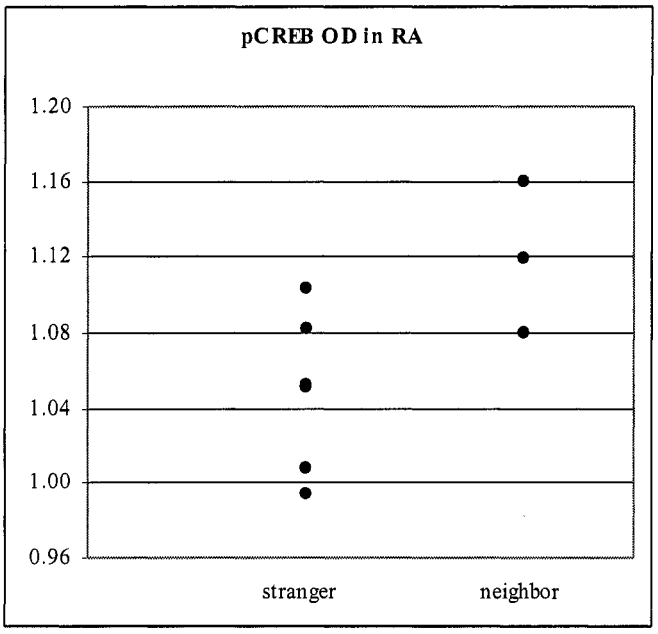

Figure 2.3: Individual pCREB OD ratios of RA/arcopallium

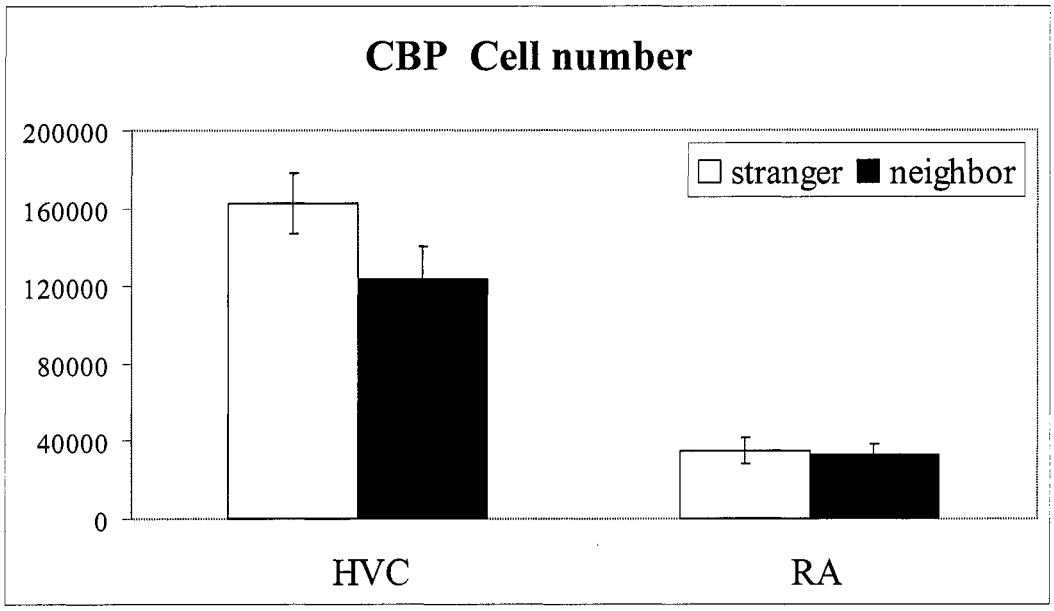

Figure 2.4: Number of cells immunolabelled for CBP in HVC and RA (Mean ± SEM)

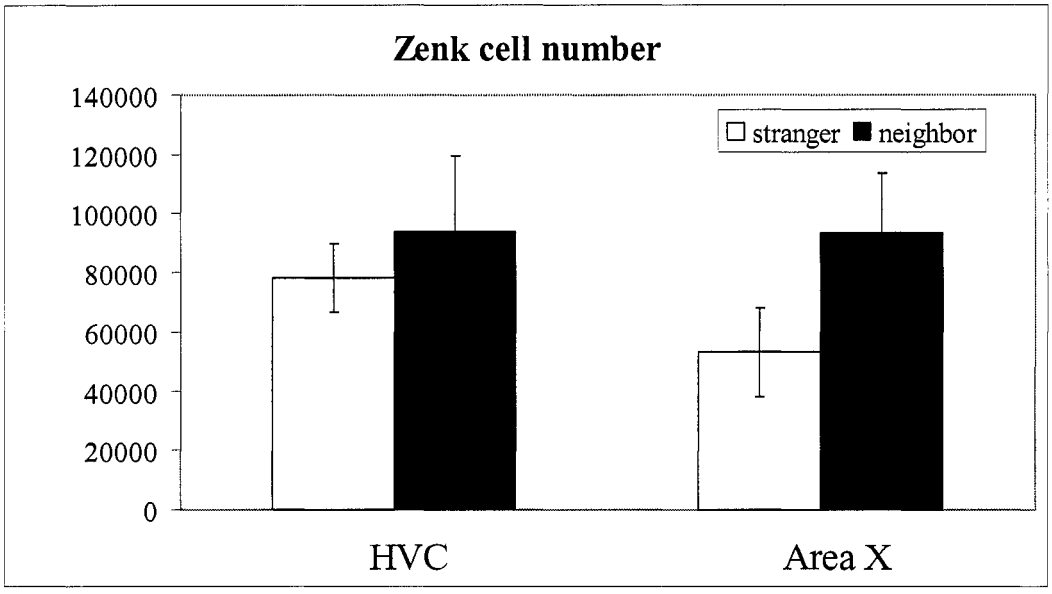

Figure 2.5: Number of cells immunolabelled for ZENK in HVC and Area X (Mean ± SEM)

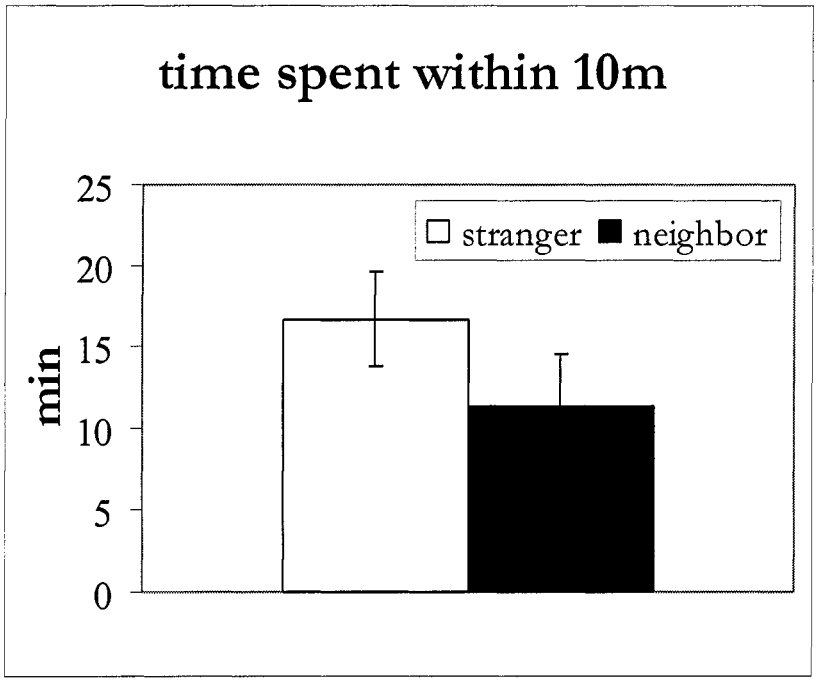

Figure 2.6: Time spent with 10 meters of the playback speaker during the 30 minute trial (mean ± SEM).

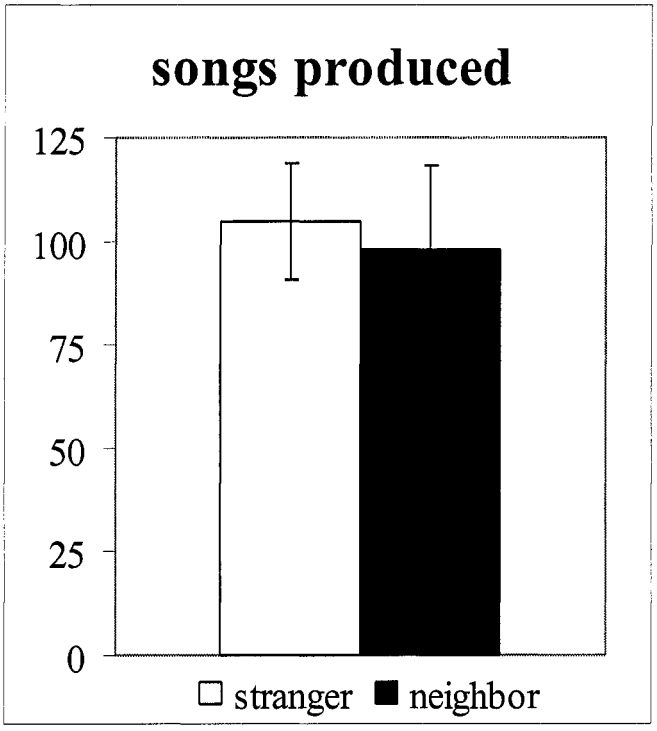

Figure 2.7: Number of songs produced during the 30 minute playback trial (mean ± SEM).

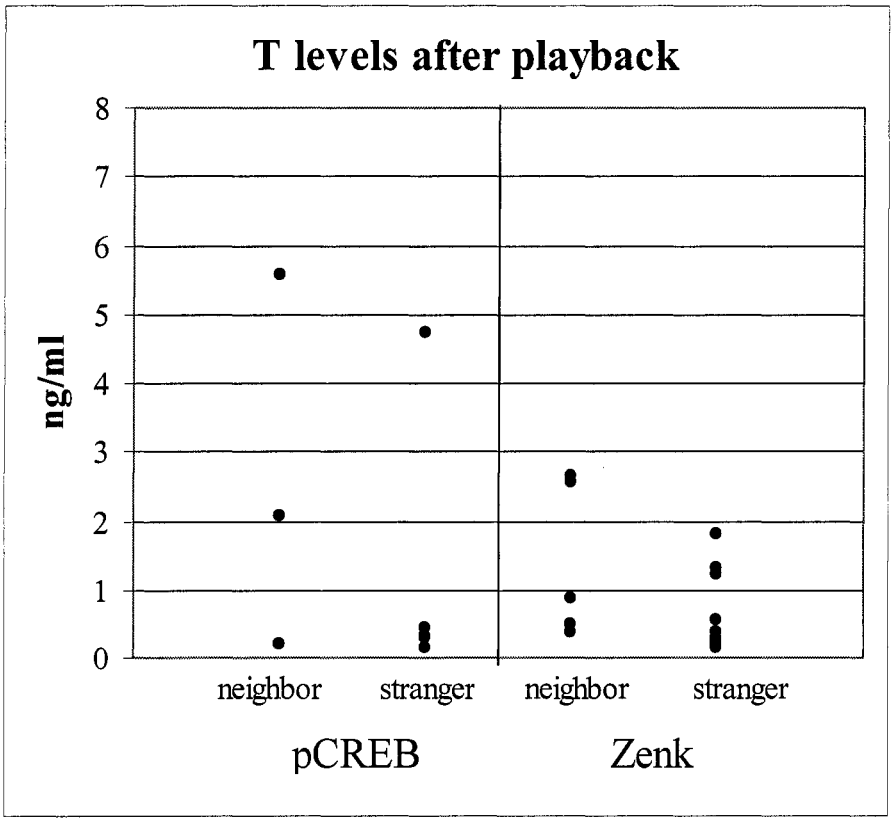

Figure 2.8: Individual circulating plasma T levels taken following the 30 minute playback in birds analyzed for pCREB and ZENK

CHAPTER III: CREB phosphorylation in HVC and CMM in response to novel or familiar songs in adult female song sparrows

INTRODUCTION

The ability to discriminate and recognize male song contributes to mate choice decisions in many female songbirds (Searcy and Yasukawa, 1996). Females must accurately assess song to help determine the quality of a potential mate. Evidence from copulation solicitation studies suggest that females song sparrows learn to identify the specific song types of their mates (O'Loghlen and Beecher, 1997). Female songbirds do not typically sing, but have the same neural song control system as males. The oscine song control system is a discrete network of nuclei involved in song behavior and is an excellent model for investigating the neural basis of learning and memory (Brenowitz et al., 1997). Female song recognition provides an opportunity to investigate the role of the song control system in recognition.

Nuclei of the song control system in the non-singing female brain are much smaller in volume than in the male brain (Nottebohm, 1976), although the extent of the sex difference depends on whether and how much the female sings (Brenowitz, 1997). Despite the difference in volume in non-singing females, the nucleus HVC (used as proper name) demonstrates complex auditory responses (Williams and Nottebohm, 1985; Whaling et al., 1997) and lesions to HVC disrupt song perception (Brenowitz, 1991; Del Negro et al., 1998; Gentner et al., 2000). Auditory information, including song, reaches HVC indirectly via forebrain auditory regions. Field L is characterized by a large number of

auditory units with a low degree of selectivity (Muller and Leppelsack, 1985), indicating that song recognition occurs somewhere downstream of Field L. There are five subregions of Field L (L, L1, L2a, L2b, L3) based on differences in cytoarchitecture and connectivity (Fortune and Margoliash, 1992; Vates et al., 1996). Anterograde tracers injected into Field L labeled cells throughout mesopallium (Wild et al., 1993). The Field L subregion L2a projects to secondary auditory areas caudal mesopallium (CM) and caudal medial nidopallium (NCM) (Vates et al., 1996). CM projects to the nucleus interface of the nidopallium (NIf), which then projects to HVC (Vates et al., 1996; Janata and Margoliash, 1999; Cardin and Schmidt, 2004). In the caudal-medial portion of mesopallium (CMM), there is an increase in auditory selectivity after learning to recognize a song as part of an operant conditioning task in European starlings (*Sturnus vulgaris*) (Gentner and Margoliash, 2003). The increased selectivity suggests that mechanisms of auditory selectivity exist within CMM (Muller and Leppelsack, 1985). Electrolytic lesions to CMM caused female zebra finches (*Taeniopygia guttata*) to lose their ability to discriminate between conspecific and heterospecific song (MacDougall-Shackleton et al., 1998). It its not clear whether the failure to discriminate is the result of disrupting a song specific processing mechanism or a more general auditory processing mechanism.

Auditory areas within the songbird brain have also been mapped using immediate early gene (IEG) activation in response to song, in particular with ZENK (the avian homologue of and an acronym for zif-268, egr-1, NGFI-A, and Krox-24). ZENK expression increases in CMM and NCM when male zebra

finches or canaries hear conspecific song (Mello et al., 1992; Mello and Clayton, 1994). ZENK expression in CMM and NCM also increases when a male zebra finch hears himself sing, but not when deafened and singing (Jarvis and Nottebohm, 1997). ZENK expression in NCM and the most caudal-medial portion of mesopallium of female European starlings increased in response to sexually relevant variation in male song (Gentner et al., 2001; Sockman et al., 2002). These results point to the role of auditory areas outside of the song control system in females learning to recognize male song. It is unknown whether these areas act alone or in conjunction with the song control system, in particular HVC, to mediate song recognition.

In this study we mapped auditory areas based on the phosphorylation of the transcription factor, cAMP response element binding protein (CREB). Phosphorylated CREB (pCREB) is necessary for the formation of long-term memories (Deisseroth et al., 1996; Guzowski and McGaugh, 1997; Stanciu, 2001; Warburton et al., 2005). CREB phosphorylation is thought to mediate the transcription processes early in long-term memory formation that stabilize synaptic changes triggered during learning (Matynia et al., 2001, see chapter 2).

Sakaguchi et al. (1999) reported for zebra finches that there was a greater number of cells immunolabelled for pCREB in HVC in response to playbacks of conspecific song than to either heterospecific song (canary) or white noise. However, no behavioral observations of the zebra finches were made during the playback and it is therefore difficult to interpret the result in a functional context. Male song sparrows on their territory during the breeding season, exposed to

novel-stranger song exhibit an increase in CREB phosphorylation in HVC relative to males hearing familiar-neighbor song (Reeves et al., 2004, chapter 2). This result suggests that long term memories of song are formed in HVC. Female song sparrows also have the ability to discriminate behaviorally between novel and familiar song (O'Loghlen and Beecher, 1997). It is unknown if females are using their smaller HVC to form auditory memories of song or if song memories are formed outside the song system.

We hypothesized that if HVC and CMM are involved in long-term memory formation of song, levels of pCREB would increase in HVC and CMM in females played novel song relative to females played familiar song. We tested this hypothesis in female song sparrows captured in the wild, most of them captured as nestlings and the rest at an unknown age. With the exception of song the females may have heard while in the nest, we knew all songs to which the females captured as nestlings and raised in the laboratory had been exposed. Thus, we could compare the role of CREB phosphorylation in auditory memory formation in HVC and CMM in response to playback of novel or familiar song.

METHODS

Subjects

Sixteen female song sparrows were collected from the wild. Four were collected in the Fall of 2001 in Sunnyside, WA (46°N, 120°W). They were of unknown age and breeding experience when captured. The other 12 female song sparrows were collected from nests in Seattle, WA (47°N, 122°W) when they were 4-5

days old. All birds were collected by permission of the United States Fish and Wildlife Department.

Song Exposure in laboratory-raised females

Laboratory-raised birds were moved to individual sound attenuation chambers between post-hatch day 30-40 and tutored with adult male song sparrow song as part of a song learning experiment (Beecher et al., in prep). All birds were tutored with songs by an interactive computer playback program throughout the summer until September 1st, 2003, when females were moved to individual cages within visual and acoustic contact of each other. The tutoring continued in the Fall with the same song stimuli.

Photoperiod and experimental history

In the spring of their second year, the laboratory-raised females received a silastic estrogen (E_2) implant (I.D. = 0.762mm, O.D. = 1.8mm, length = 12 mm) and were tested for song preference based on copulation solicitation displays. All birds failed to solicit to the playback. A control group of wild caught females, in contrast to the laboratory-raised birds, solicited to the songs suggesting that the females' willingness to solicit was likely due to a lack of breeding experience. The following summer the laboratory-raised females were in aviaries housed in vocal and visual contact with adult males on a natural photoperiod, and some of the females laid eggs. With this indication of their fecundity and evidence that prior experience with photostimulation enhances reproductive development within songbirds (Sockman et al., 2004), a second attempt to investigate their song preference was made.

Birds continued on natural daylength until Dec 1st, 2004 when they were photoshifted to 16 hours of light simulating breeding season conditions. Birds received E_2 implants and were moved to the sound attenuation chamber in which they would be tested on the same day. We gave each female a 12 mm silastic implant (see above) that raised plasma E_2 levels to the range typical of wild breeding song sparrows (Wingfield and Goldsmith, 1990). Due to space limitations, birds were randomly assigned to three cohorts At least one of the wild-raised females was assigned to each cohort. The first cohort (n = 6) received E_2 implants and moved into the sound attenuation chamber on December 20th, 2004. Birds were acclimated to the chamber for 20-22 days before copulation solicitation testing. The second cohort (n = 5) received E_2 implants on January, 4th 2005. We moved the second cohort into the sound attenuation chambers on January, 11th, 2005 where they acclimated for 7-9 days before behavioral testing. The third cohort (n = 5) received E_2 implants on January 11th, 2005 and were moved into sound attenuation chambers on January 20th, 2005. Birds acclimated for 10-11 days before behavioral testing.

Hormone samples

We took blood samples from all birds between November 27th-30th to ensure that all females had regressed levels of plasma E_2. We took another blood sample after photoshifting and 1-2 days prior to receiving an E_2 implant. We took a final blood sample after the song playback. 400 µl of blood was collected by alar venepuncture into heparinized Microtainer plasma separator tubes (Becton

and Dickinson Company, New Jersey). Blood was centrifuged and the plasma was separated and stored at –20°C and measured using steroid radioimmunoassay.

Playback

Females were isolated in the sound attenuation chamber for 24-48 hours. The females were presented with a 30 minute playback of either familiar song or novel song. The familiar song playback consisted of the computer tutor, same age tutees that learned from the computer tutor, and adult song sparrows that the females were housed within auditory and visual contact of for several months. Half of the females heard 30 minutes of novel song sparrow song recorded from the Skagit Wildlife Reserve, Mount Vernon, WA. All song stimuli were filtered using the software Syrinx (J. Burt, www.syrinxpc.com). Behavioral observations were made during the playback via a video camera in the sound attenuation chamber.

Immediately following the 30 minute playback, birds were removed from the sound attenuation chamber. A blood sample was taken within 3 minutes of the end of the playback. The birds were deeply anesthetized by methoxyflurane inhalation and perfused through the heart with heparinized 0.75% saline followed by 4% paraformaldehyde. Brains were post-fixed in 4% paraformaldehyde. Brains were embedded in gelatin and immersed for 24 hours in 20% sucrose: 10% neutral buffered formalin at 4°C. Brains were frozen on dry ice and stored at -70° C until sectioning.

Morphometry and Histology

We sectioned brains at 40 μm on a freezing microtome and stored sections at 4° C in 0.75% saline. Every third section was stained with thionin in order to locate HVC and CMM. Sections containing regions of interest were immunolabelled for pCREB within 3 days of sectioning. Endogenous peroxidase activity was quenched by a 10 minute treatment with 0.3% H2O2 in PBS-TX (0.1 M PB + 0.9% NaCl + 0.2% Triton X-100 [Sigma]), tissue was washed and blocked with 10% normal goat serum (NGS) in PBS-TX, and primary antibody for pCREB (gift of Dr. Mark Montiminy, 1:2000) was applied overnight. Sections were washed in PBS-TX, incubated in biotinylated anti-rabbit secondary antibody (Vector Laboratories, 1:200) for an hour, rinsed in PBS, incubated in avidin-biotin complex (ABC; PK-4000; Vector) for 45 minutes, rinsed in PBS, incubated with a biotinylated tyramide amplification reagent (NEN) for 10 minutes, rinsed in PBS, incubated again in ABC for 30 more minutes, rinsed in PBS, and rinsed in 0.05M Tris for 5 minutes. The color reaction was performed with 3,3' diaminobenzidine (DAB) and allowed to develop 5-20 minutes. Sections were washed in Tris, mounted onto gelatin-coated slides in distilled water and air-dried overnight, after which they were dehydrated in an ethanol series, cleared in xylene, and coverslipped.

Sections stained with thionin were projected onto a piece of paper (46X) and the Nissl defined borders of HVC and CMM were traced to estimate their volume (Brenowitz and Smith, 1997; Smith et al., 1997). CMM was defined in coronal sections as caudal to the point where the lateral ventricle is parallel to lamina mesopallius (LaM). The medial extent was from the midline to 2 mm

laterally . The caudal extent of CMM extended to the point where LaM intersects the lateral ventricle. These architectural borders were defined based on electrophysiological recordings (Muller and Leppelsack, 1985; Gentner and Margoliash, 2003; Grace et al., 2003), IEG expression (Mello and Clayton, 1994; Jarvis et al., 1998; Gentner et al., 2001; Sockman et al., 2002; Hernandez and MacDougall-Shackleton, 2004), and tract tracing experiments (Vates et al., 1996). We scanned the tracings into a computer and measured area using NIH image 2.08 (Wayne Rasband, National Institutes of Health, Bethesda, MD). The volume of each region was calculated using the formula for a cone frustum (Smith et al., 1997).

Sections immunolabelled for pCREB were magnified and captured using NIH image (v. 2.08) through a video camera mounted onto a microscope. Sampling boxes (0.086 x 0.057 mm^2) were randomly created within the region of interest in NIH image and pCREB immunolabelled cells were counted. There is not a clear lateral boundary of CMM, therefore it was divided into four 500 μm wide bins along the medial-lateral dimension, with the midline as the zero point. Counting boxes were randomly drawn over CMM and at least 2 counting boxes that fell completely within each 500 μm bin were chosen. The 2 mm span represented the furthest distance from the midline that auditory responses had been recorded or described in mesopallium (Muller and Leppelsack, 1985). Density of cells was calculated by dividing the number of cells counted by the number of randomly generated sampling boxes. Cell number was estimated by multiplying the density and the volume measured.

Steroid Radioimmunoassay

Plasma samples were equilibrated overnight with approximately 2000 cpm of tritiated 17β-estradiol. The following day E_2 was extracted from the plasma using dichloromethane. The extract was evaporated and dissolved in ethyl acetate/iso-octane. Bound and unbound E_2 was separated by adding 0.5 ml of dextran-coated charcoal for 12 minutes followed by centrifugation at 2000 rpm for 10 minutes. The supernatant, containing bound E_2, was decanted into scintillation vials and mixed with 4.5 ml of Ultima Gold scintillation fluid (Packard Instrument, Meriden, CT, USA). Samples were counted for 5 minutes in a Beckman LS 3801 liquid scintillation counter. A standard curve was generated with known amounts of steroid and the percentage of labeled steroid bound to the antiserum was calculated. The intra assay variability was 2.2% CV and the detection limit was 0.07 ng/ml. All samples were assayed together in one E_2 assay to eliminate effects of inter-assay variation.

Plasma luteinizing hormone (LH) was measured with a double-antibody, post-precipitation radioimmunoassay (Wingfield et al., 1991). The assay uses purified chicken LH as standard and iodination by the chloramine T method, and successfully measures LH in a wide variety of songbirds. All samples in this study were measured in a single assay, for which the intra-assay coefficient of variation was 4.9% and the detection limit was 0.039 ng/ml.

RESULTS

Neurons were clearly immunolabelled in HVC and CMM in ten of the sixteen subjects and statistical analyses of pCREB only include these birds.

Levels CREB phosphorylation in HVC in response to novel or familiar songs were compared using a one-way ANOVA. There was no difference in the number ($F_{(9)}$=1.114, p=0.319) or density ($F_{(9)}$=0.825, p=0.387) of pCREB immunolabelled cells in HVC between the two playback groups (Figure 3.1). There was one outlier, GR, within the novel song group with a very high number of pCREB immunolabelled cells in HVC (Figure 3.2). The outlier was wild-raised and potentially had breeding experience before being brought into the laboratory. GR also solicited for copulation in response to the playback 104 times in 30 minutes, whereas none of the other females analyzed for CREB phosphorylation exposed to novel song solicited in response to the playback. There was one wild-raised female in the familiar playback group. She exhibited low levels of pCREB in HVC as the laboratory-raised females did and only solicited to the playback on one occasion.

In order to investigate the prediction that the caudal and medial most regions of mesopallium are involved in song recognition CMM was divided and analyzed in several different ways (as described in Gentner et al., 2001). Images of CMM were first divided into a grid to determine if predicted regional differences of CREB phosphorylation were evident (Figure 3.3). The volume of each sector within the grid was calculated by measuring the area in adjacent Nissl stained sections and generating a volume based on the formula for a cone frustrum. The ends of the cone frustrum were subtracted from the total volume and the remaining value, the volume between the two adjacent sections, we defined as a sector. Cell density for each sector was multiplied by the sector

volume to generate an estimate of cell number. Not every bird sampled was represented in every sector. Values of cell number for each sector within the grid were compared with a one-way ANOVA with playback group as the independent variable and each sector within the grid as a dependent variable. There were no differences detected and the analysis did not reveal any discernable variation of CREB phosphorylation in CMM (for all tests, $p > 0.05$).

Due to the small sample size and random sampling scheme, many comparisons had a very low degree of statistical power. Data for sectors within the grid were therefore combined so each sector of the grid was 240 μm x 500 μm. A one-way ANOVA once again failed to reveal any difference in the number of cells immunolabelled for pCREB between the two playback groups (for all tests, $p > 0.05$).

Another approach was taken to determine if a pattern of CREB phosphorylation existed in CMM. Based on the assumption that new memories are not being formed in the familiar song group, we expect those values to represent a baseline of CREB phosphorylation. If CREB phosphorylation is increasing in response to novel song, it should increase relative to the average amount of pCREB found in response to familiar song. We calculated the mean number of immunolabelled cells across all sectors within the grid in the familiar song group. A one-sample t-test compared each sector in the grid of the novel song group to the mean cell number from the familiar group. Seven of the 45 boxes from the novel group showed a higher number of pCREB immunolabelled

cells. There was not a discernable pattern across the grid or high levels of pCREB in the caudal-medial region of CMM as predicted.

In order to determine if the medial portion of CMM is involved in song recognition, images of CMM were divided into four columns to determine if pCREB levels in response to novel song were higher in medial portions of mesopallium. Each column is 500 μm wide and the length is defined by the rostral and caudal extents of CMM (see Figure 3.4A) Right and left hemisphere columns were compared using a paired samples t-test and no differences were detected (Table 3.1). Therefore, the number of immunolabelled cells in each column was combined across hemispheres. There was no difference in CREB phosphorylation between the novel and familiar playback groups in any of the four columns (for all tests, $p > 0.05$; Figure 3.5). Levels of pCREB in CMM columns of bird GR, the wild-raised outlier in HVC, were within the range of other females measured (Figure 3.6)

In order to determine if the caudal portion of CMM is involved in song recognition, images of CMM was divided into rows (Figure 3.4 B). The thickness of the rows was defined by the distance between immunolabelled sections. Each row was 120 μm thick and 2 mm wide. Height was determined by the distance between the lateral ventricle and LaM (see Figure 3.4 B). Eleven right and left hemisphere rows were compared using a paired sample t-test (Table 3.2). One of the rows was significantly different between hemispheres ($t_{(4)} = 10.87$, $p = 0.00$), so data were not combined across hemisphere for that row. Data collected from all other rows were combined across hemispheres. There was no difference in

CREB phosphorylation between playback groups in all rows (for all tests, $p > .05$; Figure 3.7). Levels of pCREB in CMM rows of bird GR, the wild-raised outlier in HVC, were within the range of the other females measured (Figures 3.8).

After photoshifting and receiving E_2 implants all 16 birds had plasma E_2 levels as high or higher than observed in wild female song sparrows on their territory during the breeding season (see Figure 3.9,Wingfield and Goldsmith, 1990). A one-way ANOVA was used to compare plasma E_2 and LH levels between the two playback groups. Plasma E_2 levels ($F_{(9)} = 0.475$, $p = 0.502$) and plasma LH ($F_{(9)} = 2.195$, $p = 0.164$) following playback did not differ between the two playback groups.

DISCUSSION

Female song sparrows did not show selective CREB phosphorylation in response to novel or familiar playback of conspecific song in HVC or CMM. The absence of a selective CREB phosphorylation in response to novel or familiar song in females differs from the finding in male song sparrows (Reeves et al., 2004, chapter 2). The difference could be explained by the lack of behavior in response to the playback in the females or the artificial environment in which the experiment was executed. Only two of the ten females that were analyzed for pCREB in HVC responded to the playback with a copulation solicitation. Although the behavioral repertoire of a female song sparrow is certainly not limited to copulation solicitation, the females did not exhibit any other soliciting behaviors like chitter calls or aggressive responses like wing waving that would have indicated they were reacting to the playback. Secondly, the absence of a

selective pCREB response may be because the experiment was conducted in an artificial environment. Although several studies have successfully measured copulation solicitation (Vallet and Kreutzer, 1995; O'Loghlen and Beecher, 1997; Draganoiu et al., 2002) and auditory processing (Mello et al., 1992; Mello and Clayton, 1994) in songbirds, the selective pCREB response in males was measured during the breeding season in males defending their territory in response to song playbacks. It is possible that songbirds will only form long term memories of song in biologically relevant situations.

The laboratory-raised females in this study showed no behavioral response to the playback of either novel or familiar song. The hormone assays suggest that plasma hormone levels in all females were adequate to induce copulation solicitations in comparison to plasma levels measured in free-living female song sparrows during the breeding season (Wingfield and Goldsmith, 1990) The females may have become desensitized to playback of song in the sound attenuation chamber. The laboratory-raised females were raised in identical chambers and heard song playback throughout their natal summer. Hearing song for so long without any behavioral contingencies may have desensitized the females to the playback to the point that they would no longer respond to song in the chamber, regardless of their seasonal or hormonal state. In a more recent experiment, laboratory-raised females that were raised in social groups and had no prior experience with the sound attenuation chamber did solicit to song playback in the chamber (O'Loghlen, personal observations).

The levels of pCREB in HVC in the two wild-raised females fit our hypothesis based on the result in males (Chapter 2). Given the small sample size it is not possible to draw any conclusions from this data. Also, the difference in behavioral response to the playback between the two wild-raised females introduces another variable. The female that heard novel song solicited to the playback 104 times, whereas the female that heard familiar song only solicited 1 time. It is not possible to determine if the low level of pCREB in HVC in the second female was related to her lack of response or because she was not forming long term memories of previously learned song types. In order to properly investigate the role of pCREB in forming long-term memories of song in female HVC, wild-raised females should be used to compare the effect of novel or familiar (i.e. mate) song.

Table 3.1: Paired Samples Test comparison of CMM divided into columns from the right and left hemispheres

	Paired Differences		t	df	Sig. (2-tailed)
	Mean	Std. Error Mean			
Right 1 – Left 1	1229.461	-1337.6294	-1.09	8	0.31
Right 2 – Left 2	807.7234	-98.2529	-0.12	8	0.91
Right 3 – Left 3	1457.385	-561.1692	-0.39	8	0.71
Right 4 – Left 4	1874.239	-1662.5437	-0.89	8	0.40

Table 3.2: Comparison of CMM divided into rows from the right and left hemispheres

	Mean	**Std. Dev**	**SEM**	**t**	**df**	**Sig. (2-tailed)**
RIGHT-LEFT 3	824	4816	2781	0.30	2	0.79
RIGHT - LEFT 4	-1906	1118	646	-2.95	2	0.10
RIGHT - LEFT 5	-1090	1214	607	-1.80	3	0.17
RIGHT - LEFT 6	4375	9431	4218	1.04	4	0.36
RIGHT - LEFT 7	-3213	4975	2488	-1.29	3	0.29
RIGHT - LEFT 8	-1453	299	134	-10.87	4	*0.00*
RIGHT - LEFT 9	161	863	432	0.37	3	0.73
RIGHT - LEFT 10	248	1418	819	0.30	2	0.79
RIGHT - LEFT 11	-2488	2084	1203	-2.07	2	0.17
RIGHT - LEFT 12	-43	4699	2102	-0.02	4	0.98
RIGHT - LEFT 13	-1751	3715	2145	-0.82	2	0.50

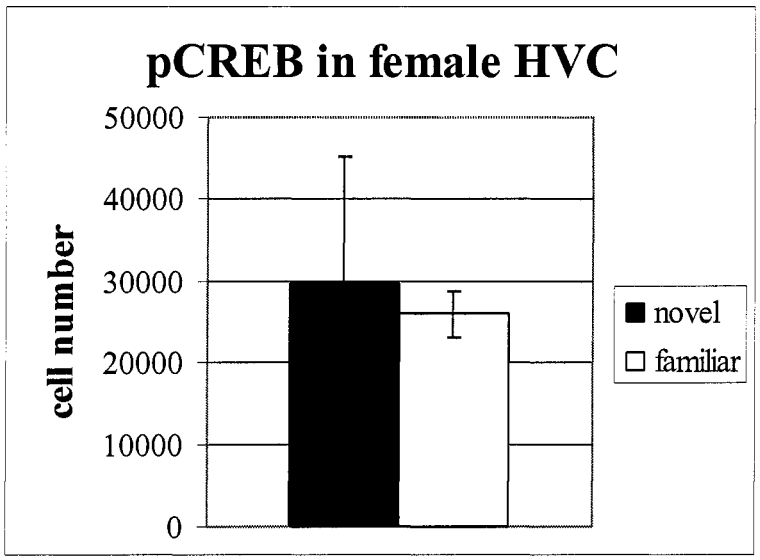

Figure 3.1: Mean (+SE) number of cells immunolabelled for pCREB in HVC

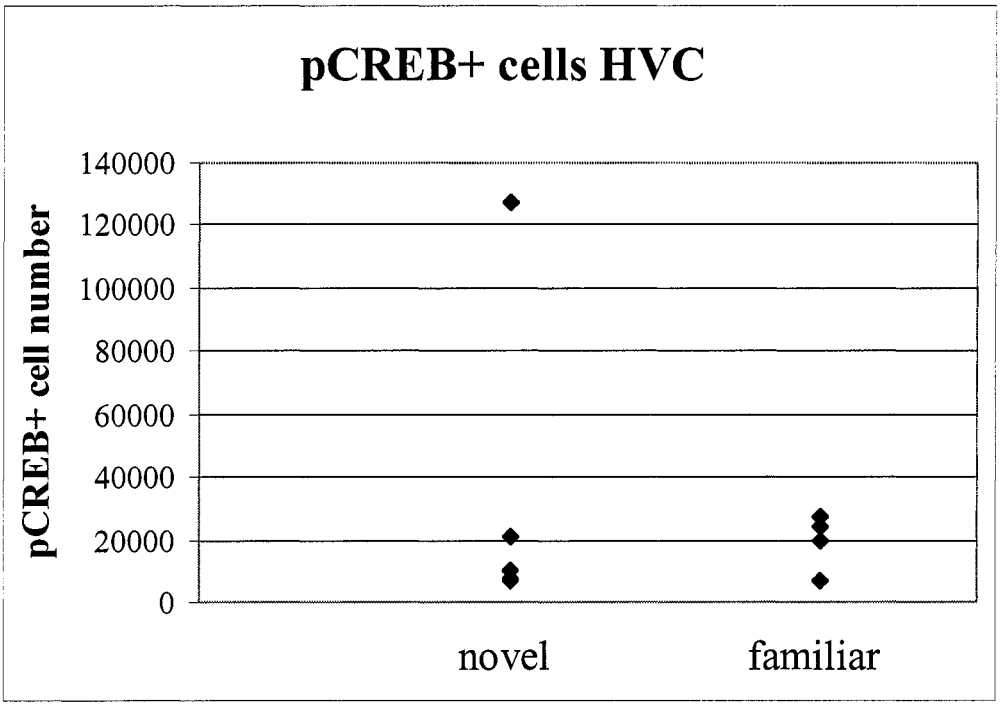

Figure 3.2: Number of cells immunolabelled for pCREB in HVC for each female. The outlier in the novel playback group was raised in the wild.

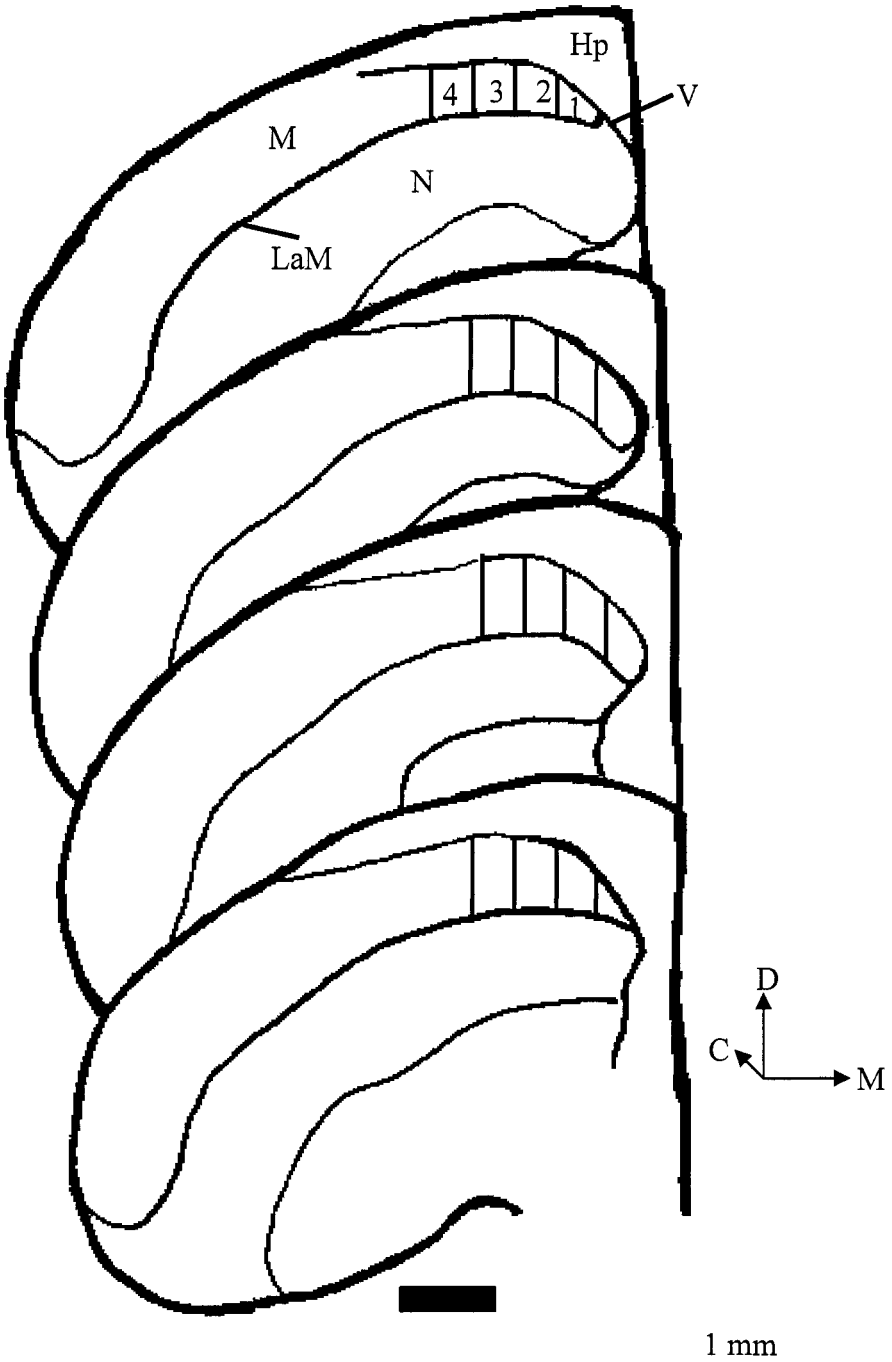

Figure 3.3: Serial reconstruction of coronal sections sampled to measure number of cells immunolabelled for pCREB in CMM. Numbers depict sectors within CMM that were sampled. N = Nidopallium; LaM = Lamina mesopallius; CMM = Caudal Medial Mesopallium; M = Mesopallium; Hp = Hippocampus; V = Lateral ventricle.

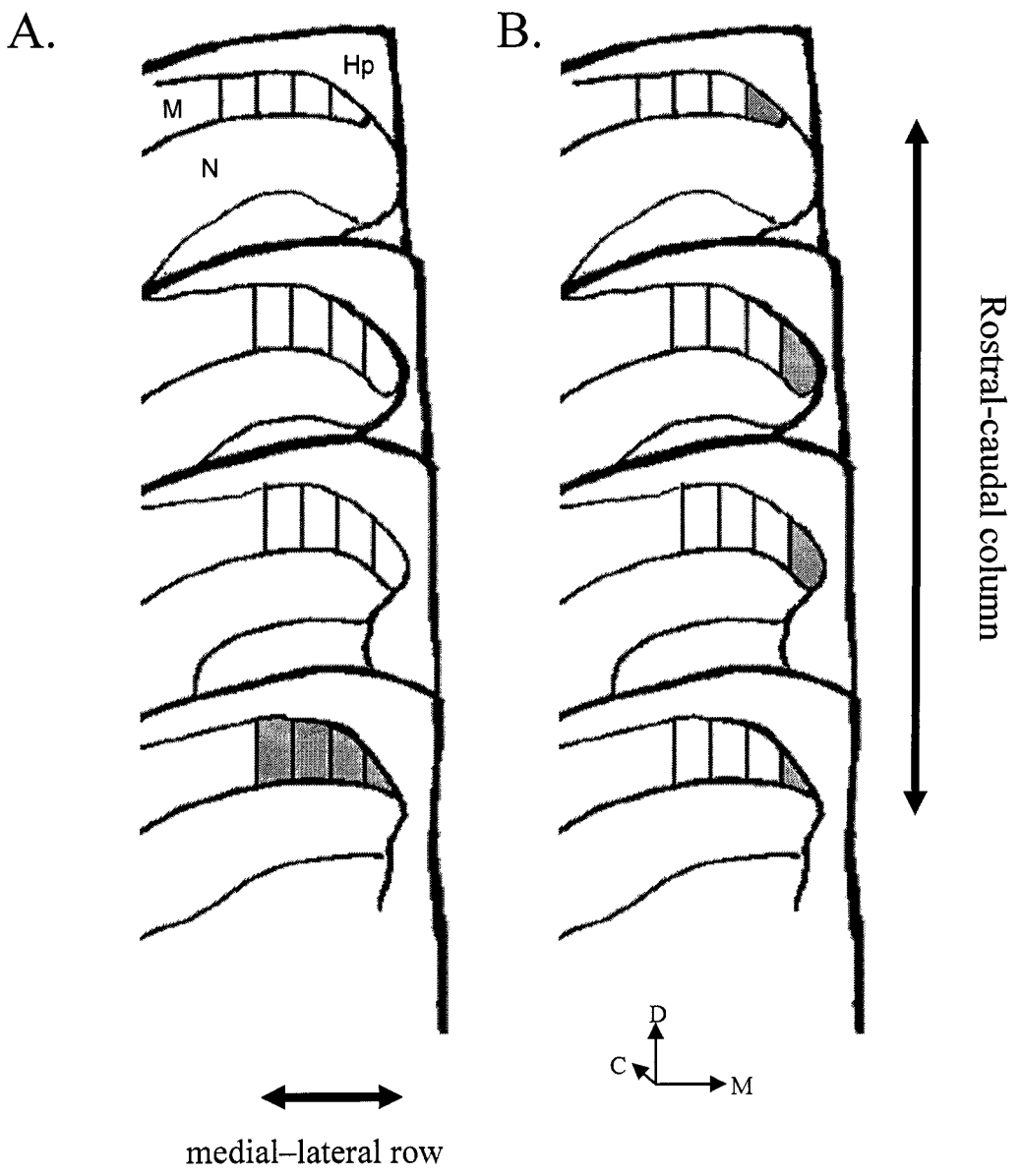

Figure 3.4: Serial reconstruction of coronal sections sampled to measure number of cells immunolabelled for pCREB in CMM in the rostral-caudal and medial-lateral dimension. A. Shaded area depicts a medial-lateral row, data from sectors within the row were combined to test hypothesis that CREB phosphorylation increases in more caudal rows in response to stranger song. B. Shaded area depicts a rostral-caudal column, data from sectors along the midline were combined to test the hypothesis that CREB phosphorylation increases in the medial portion of mesopallium. N = Nidopallium; M = Mesopallium; Hp = Hippocampus.

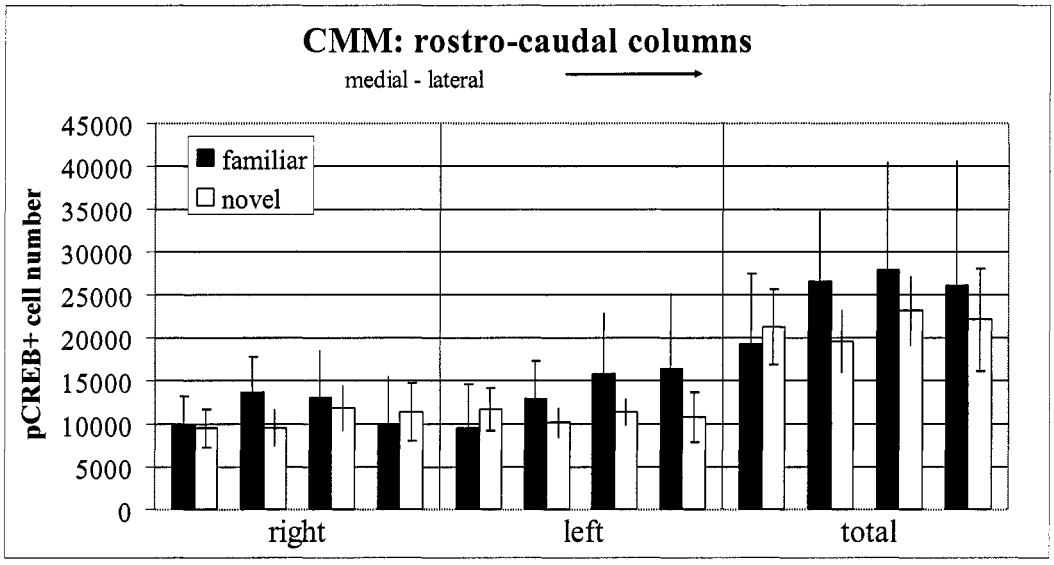

Figure 3.5: Mean (+SE) number of cells immunolabelled for pCREB in four columns of CMM.

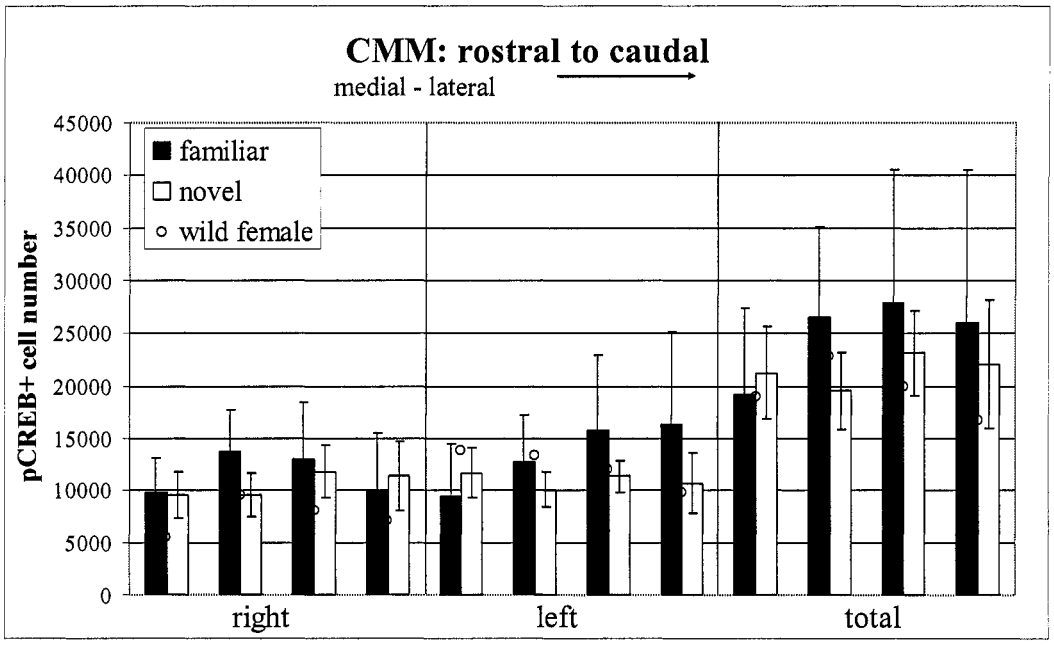

Figure 3.6: Mean (+SE) number of cells immunolabelled for pCREB in four columns of CMM. Open circles depict the wild-raised female, GR, that exhibited much higher levels of pCREB in HVC.

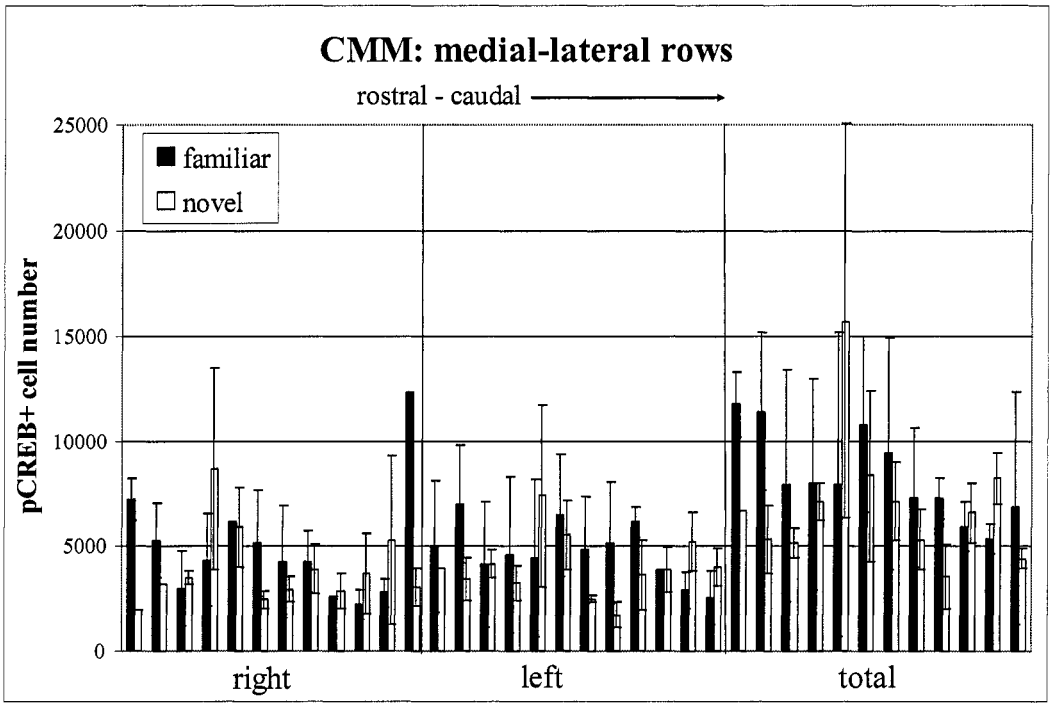

Figure 3.7: Mean (+SE) number of cells immunolabelled for pCREB in 13 rows of CMM.

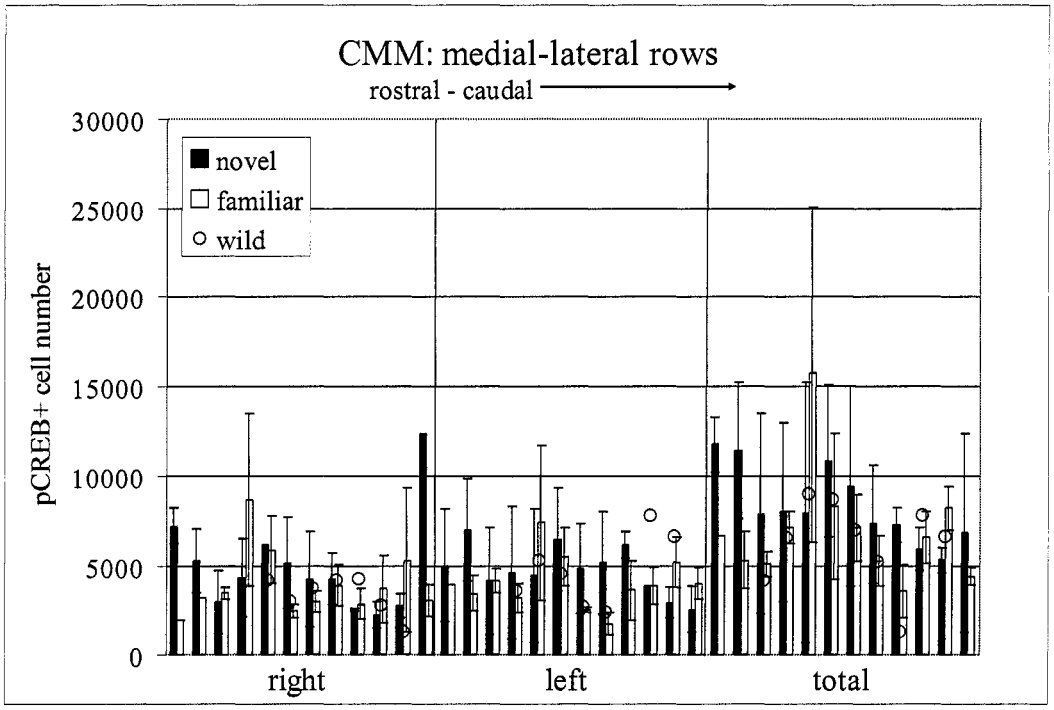

Figure 3.8: Mean (+SE) number of cells immunolabelled for pCREB in 13 rows of CMM. Open circles depict the wild-raised female, GR, that exhibited much higher levels of pCREB in HVC.

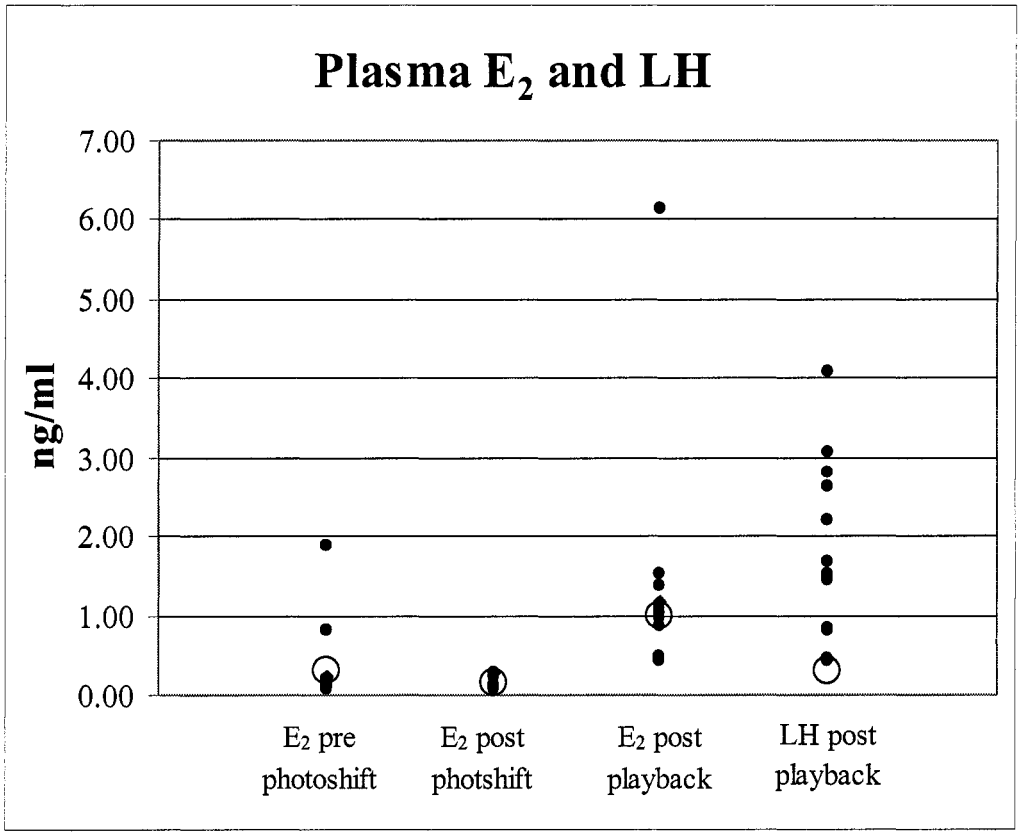

Figure 3.9: Individual circulating plasma E_2 levels on short days before photoshifting to 16L:8D, on long days before receiving a E_2 implant, and E_2 and LH after the 30 minute playback period. Open circles depict the wild-raised female, GR, that exhibited much higher levels of pCREB in HVC.

CHAPTER IV: Lesions to the anterior medial forebrain affect neighbor/stranger discrimination in wild male song sparrows

INTRODUCTION

The anterior forebrain pathway (AFP) of the oscine song control system is believed to be necessary for song learning and perception. The AFP consists of projections from nucleus HVC to Area X, a basal ganglia homologue, and then to the dorsal lateral nucleus of the medial thalamus (DLM). DLM projects to the lateral portion of the magnocellular nucleus of the anterior nidopallium (lMAN). The nucleus lMAN projects both to the motor pathway via the robust nucleus of the arcopallium (RA) and to Area X. During development, lesions to the AFP have dramatic effects on song learning (Bottjer et al., 1984; Sohrabji et al., 1990). Once birds reach adulthood however, lesions to the AFP have only subtle effects on the production of crystallized song (Scharff, 1991; Williams and Mehta, 1999; Brainard and Doupe, 2001). There is also evidence to suggest that the AFP processes auditory information. Single unit extracellular recordings of lMAN neurons show strong auditory responses that are primarily selective to the bird's own song (BOS) (Doupe and Konishi, 1991). LMAN apparently acquires BOS selectivity in conjunction with song learning (Solis and Doupe, 1997). Many of the neurons in lMAN that respond selectively to BOS also respond, but less selectively, to tutor song and conspecific song (Solis and Doupe, 1999).

Lesions to the anterior forebrain pathway reduce a songbird's ability to make perceptual discriminations in operant conditioning tasks (Scharff et al., 1998; Burt et al., 2000). Burt et al. (2000) lesioned lMAN in female canaries and

tested the bird's ability to discriminate between previously learned pairs of conspecific songs, heterospecific songs, frequency modulated tones, and a pure tone vs. white noise. Bilateral lesions to lMAN disrupted their ability to perform all of these discriminations, suggesting that lMAN plays a role in the perception of complex acoustic stimuli, including songs. We investigated whether the AFP plays a role in song discriminations in wild male birds that must make conspecific song discrimination for successful territory defense.

Song sparrows *(Melospiza melodia)* discriminate between the songs of their neighbors and those of strangers (Stoddard et al., 1990; Stoddard et al., 1991). A male song sparrow without a territory (i.e. a stranger) during the breeding season will try to establish a territory from within an existing neighborhood or usurp an existing territory altogether. Song sparrows defending their territory respond more aggressively to playback of stranger song than that of a neighbor. Based on the laboratory evidence that lMAN is necessary for song discrimination, we predicted that lesions to lMAN in wild male song sparrows would impair their ability to discriminate between neighbor and stranger songs, affecting the strength of aggressive response between the two playbacks.

METHODS

Behavioral data were collected at the Skagit Wildlife Reserve in Mount Vernon, WA (48.321°N, 122.383°W). In the early spring we individually color banded the legs of 13 male song sparrows and their neighbors with a unique color combination for identification. Territory boundaries were measured based on locations from which the birds sang and defended against playback. Full song

repertoires of all birds in the study and their neighbors were recorded using a Sennheiser ME-67 directional microphone and a Sony TC-D5M tape recorder. Song recordings were digitized using the software program Syrinx (J. Burt, www.syrinxpc.com).

Songs were played to territorial birds using a Dell Inspiron 1100 laptop computer with a 16-bit sound card. A directional speaker was attached to the computer via a 20 m cable. The directional speaker was constructed using a midrange tweeter with a broad frequency range placed in a Sony parabolic reflector (Sony PBR-330). The software program Syrinx (J. Burt, www.syrinxpc.com) was used for the playback of the digitized song recordings. Syrinx displayed playable spectrograms of all playback stimuli, spectrograms depicting the repertoire of the focal bird, and a real-time spectrograph of the interaction recorded via a directional microphone. All songs played and recorded and all behavioral observations during trials were recorded into Syrinx for later analysis.

We used a one-speaker neighbor/stranger discrimination paradigm. Playback trials consisted of heterospecific, stranger, and neighbor song. The first playback acquired a baseline measure of the bird's behavior before the lesion. All playback trials began with a heterospecific banded wren (*Thryothorus pleurostictus*) song. Banded wren song was selected because its frequency range and syntactical structure are similar to song sparrows, but this species lives only in Central America. We selected stranger songs from within the same population of song sparrows, but from birds at least 200 m apart and therefore unlikely to

have interacted at territorial boundaries. Song stimuli used as stranger song for one bird were used as neighbor song for another bird. We selected a shared song type for the neighbor playback to accurately simulate communication between neighboring song sparrows (Beecher et al., 1996). The order of neighbor or stranger song playback was randomly chosen on the first trial and alternated for each bird thereafter. The focal bird's behavior was monitored for at least 10 minutes before playback began. Playback was not started if the bird was actively defending against another bird or continuing to defend in response to a previous playback. A song was played every 12 seconds for 3 minutes for a total of 15 songs. We monitored the latency of first response, number of songs produced, number of flights, number of threat displays (e.g. wing waves), the amount of time continuing to defend after the playback ended and the closest approach to the speaker.

After a playback trial, we captured the bird and brought them into the laboratory. We anesthetized the bird with isoflurane and placed it in a stereotaxic surgical apparatus. We made electrolytic lesions because previous studies showed that excitotoxic lesions of lMAN using racemic N-methyl aspartic acid (NMA) caused nonspecific damage in the tissue of brain layers dorsal to lMAN due to reflux of the NMA up the track made by the micropipette used for delivery (Burt et al., 2000). For 8 males, we directed an electrode made of tungsten wire (0.28 mm diameter) insulated with Teflon except at the tip at the following stereotaxic coordinates (relative to the intersection of the midsagittal and transverse sinuses and the brain surface): anteroposterior = 4.3 mm (rostral);

mediolateral = 1.0 mm and 1.75 mm; depth = 2.8 mm and delivered a 300 μamp current for 60 seconds. Three birds received a sham surgery procedure to control for generalized effects of capture and surgery; The top layer of the skull was opened and the electrode was lowered to the second layer of the skull and no current was delivered. After the surgery, all birds were injected with a broad-spectrum antibiotic and electrolytes to facilitate recovery. Birds recovered for at least 3 days before returning to their territory. We removed birds that had replaced the subject on their territory during their absence, and kept them in captivity for the extent of the study. All subjects but one regained their territory after release, and the second behavioral test occurred one week later. The order of playback stimuli was maintained from the pre-lesion playback trial. After the playback trials were completed, we recaptured the lesioned birds to verify the site of the lesion.

The birds were deeply anesthetized by methoxyflurane inhalation and perfused through the heart with heparinized 0.75% saline followed by 4% paraformaldehyde. We post-fixed brains in 4% paraformaldehyde and refrigerated for 24 hours at 4°C. We embedded the brains in gelatin and immersed them for 24 hours in 20% sucrose: 10% neutral buffered formalin at 4°C. We froze the brains on dry ice and stored them at -70° C until sectioning.

We sectioned brains at 40 μm on a freezing microtome and stored sections at 4° C in 0.75% saline. Every third section was stained with thionin to determine the extent of the lesion. The magnocellular neurons of lMAN are easily

distinguished from other neurons of the anteromedial portion of the nidopallium in Nissl stained sections.

RESULTS

Two of the 13 subjects were only tested on baseline playback trials. One lesioned bird was not recaptured after the post-lesion playback trial and disappeared from his territory shortly thereafter and the other failed to regain his territory. We analyzed brains of seven lesioned birds and six of the lesions missed the intended target lMAN. Only one bird received a partial lesion of lMAN. Six of the lesion sites were located caudal to lMAN and dorsal to Area X in nidopallium and mesopallium (Figure 4.1).

The six behaviors measured during the playback trial were combined using a Principal Components Analysis (PCA). The first factor contained number of flights, closest approach to the speaker, and the amount of time continuing to defend after the playback had ended and accounted for 61% of the variance. Items were weighted based on their contribution to the first factor to generate an 'aggression score'. The formula for computing a score for each trial was: (0.639 × number of flights) + (0.574 × amount of time continuing to defend) - (0.511 × closest approach).

We used a paired samples t-test to compare the bird's aggression score in response to neighbor and stranger song playback during the pre-lesion baseline trial. Bird's aggression scores were significantly higher in response to stranger song playback than neighbor song playback ($t_{(12)} = -2.38$, $p = 0.35$, Figure 4.2).

The bird's ability to discriminate between neighbor and stranger songs was impaired during the second playback after the lesion surgery. The lesioned bird's aggression score did not differ between neighbor and stranger song playback ($t_{(7)} = -0.54$, $p = 0.61$, Figure 4.2). Birds that received a sham lesion surgery also did not differ on aggression score ($t_{(2)} = -1.22$, $p = 0.35$, Figure 4.2). The lack of a significant difference in the sham lesion group is likely attributable to the small group size ($N = 3$). Also, one of the birds in the sham lesion group responded aggressively to both neighbor and stranger song playbacks in the baseline and post surgery test, but his impact was much stronger on the test of the sham lesion group because to the small sample size.

We used a paired sample t-test to compare the birds aggression score on the baseline trial to the post-lesion trial. The lesioned birds aggression score in response to the stranger song was significantly lower following the lesion surgery ($t_{(7)} = 2.32$, $p = 0.05$, Figure 4.3).

At no time did the subjects respond aggressively to the banded wren song, either before or after the lesion. This suggests that the ability to discriminate heterospecific song was not impaired in the birds that received forebrain lesions. However, the lack of a response before and after the lesion make it impossible to make any strong conclusions regarding heterospecific song discrimination.

DISCUSSION

The failure of male song sparrows to discriminate between neighbor and stranger song following lesions suggests a role of the anteromedial forebrain in song perception. However, the inaccuracy of the lesion sites makes this result

difficult to interpret. The lesions did not damage any known auditory processing regions that may have had general effects on auditory perception or nuclei of the AFP that may have had more subtle effects on song perception.

The lesions may have damaged fibers of passage within the AFP. Axons projecting from HVC to Area X in canaries travel rostrally through the medial portion of lamina mesopallius (LaM) until dorsal to Area X, where axons descend ventrally through nidopallium and medial striatum into the dorsal border of Area X (Nottebohm, 1976). Six of the seven lesion sites were dorsal to Area X, where axons descend ventrally as described by Nottebohm. However, without knowing the route of HVC to Area X axonal projections in song sparrows, it is not possible to determine if lesions described here disrupted the projection from HVC to Area X. Evidence from the zebra finch describes a different path for the projection from HVC to Area X (Bottjer et al., 1989). Bottjer et al. describe axons leaving HVC medially and traveling beneath the lateral ventricle. At the level of Area X, the axons diverge and enter from the medial side. If the HVC to Area X axonal projection in song sparrows follows the same path as in zebra finches, the lesions would not have disrupted fibers of passage within AFP.

The bird's ability to discriminate neighbor and stranger song in the baseline playback trial replicates previous findings described in the song sparrow (Stoddard et al., 1990; Stoddard et al., 1991). Following the lesion surgery, there was no difference in the bird's aggression scores in response to neighbor or stranger song. This finding suggests that birds were unable to discriminate between the two playbacks because their song perception abilities were impaired.

There was also no difference in the sham lesion group, thus we can not attribute the lack of a behavioral difference in the lesioned birds directly to the lesion.

The lesioned bird's aggression score in response to stranger song was significantly reduced following the lesion surgery. The behavioral effect measured after the lesion was not in the direction anticipated. Our original prediction, based on the AFP role in song perception, was that song sparrows unable to discriminate would respond aggressively to all song playback. For instance, HVC lesioned females solicit to heterospecific song, increasing their response despite a lack of discrimination (Brenowitz, 1991). However, the lesioned birds reduced their aggressive response to stranger song and their aggressive response was similar to a neighbor playback trial. One possible explanation is the spatial location of the speaker. If a bird is unable to make a song discrimination, he may resort to a spatial discrimination. Given the speaker was placed on the territory boundary, the bird may have attributed the song to the neighbor.

Future experiments will have to determine if the effect of anteromedial forebrain lesions described here are due to disruption of the AFP or the result of more general anterior forebrain damage. The first manipulation to do is confined lesions within nuclei of the AFP. Lesions to Area X and lMAN would determine if the behavioral effect is mediated by the song control system. A second manipulation would inject an anterograde tracer, such as DiI, a lipophilic dye, into HVC and a retrograde tracer, such as Fluro-Gold, in Area X and replicate the methods described above. The presence of DiI labeled cells in Area X or Fluro-

Gold labeled cells in HVC would illustrate whether the axonal pathway was intact. This method would also allow tracking the pathway itself and determine if the lesions described here were in the same area. Another manipulation would be to leave all of the nuclei of the AFP intact, but administer inhibitors of neuronal activity to block the activity of the AFP. HVC projection neurons to Area X are glutamatergic (Dutar et al., 1998). A glutamate receptor blocker, such asalpha-methyl-4-carboxyphenylglycine (MCPG), a metabotropic glutamate receptor (mGluR) antagonist, injected into Area X could substantially block the signal coming from HVC. Although, this manipulation would also disrupt local, although minimal, glutamatergic activity in Area X. Finally, targeted photolysis of Area X projecting neurons in HVC in zebra finches has successfully removed the Area X projecting cell type in HVC, leaving the RA projection neurons and interneurons within HVC intact and song unaffected (Scharff et al., 2000). This manipulation could provide insight into the role of AFP without any disruption of the nuclei by removing the input from HVC.

This experiment describes a powerful method of investigating the song control system's role in song discrimination and recognition. Male song sparrows defending their territory must discriminate complex auditory signals from familiar and unknown sources in order to maintain the integrity of their territory. We show here that song sparrows are able to regain their territory after a lesion surgery and a one week absence. Our results suggest a role of the anteromedial forebrain in conspecific song discrimination. More definitive claims about the role of the AFP cannot be made until we have more information about

the location of axonal connections between nuclei of the AFP and the role AFP nuclei themselves play in song discrimination.

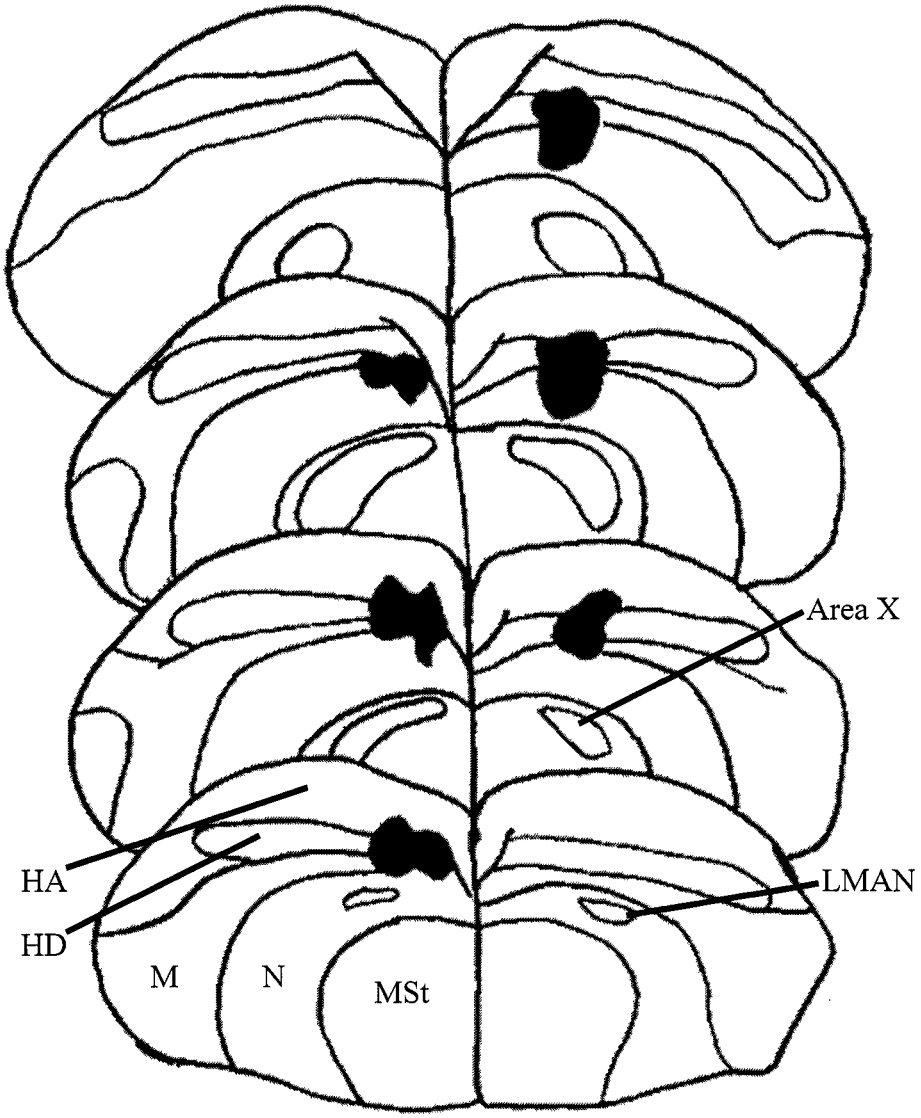

Figure 4.1: Serial reconstruction of a lesion of the anteromedial forebrain that was effective in impairing discrimination of neighbor and stranger song. Black indicates the site of the lesion. MSt = Medial Striatum; N = Nidopallium; M = Mesopallium; HD = Hyperpallium dorsale; HA = Hyperpallium apicale; LMAN = Lateral Magnocellular Nucleus of the Nidopallium.

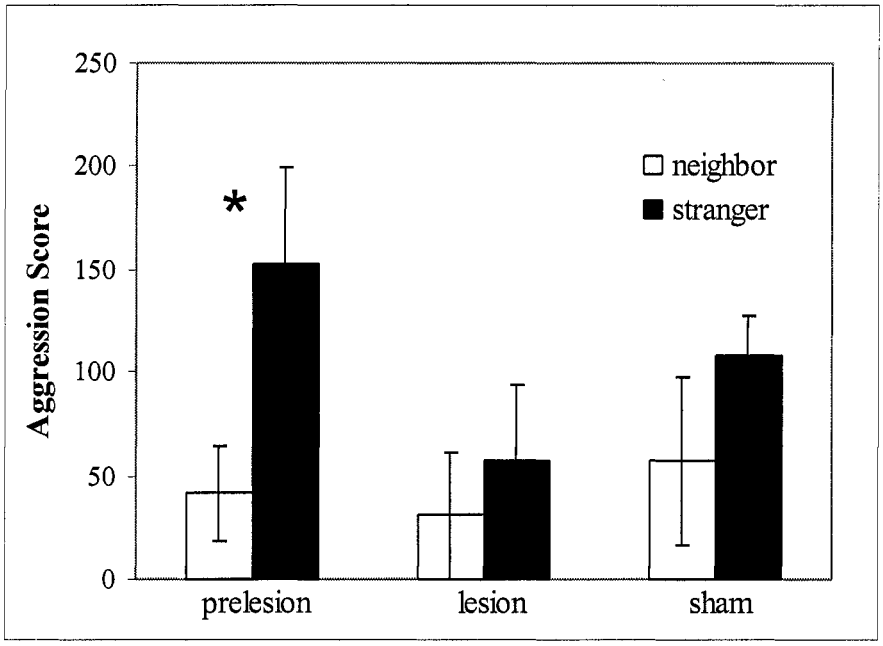

Figure 4.2. Mean (+SE) aggression score in response to neighbor and stranger playback in baseline prelesion birds, birds that received forebrain lesions, and sham lesion birds.

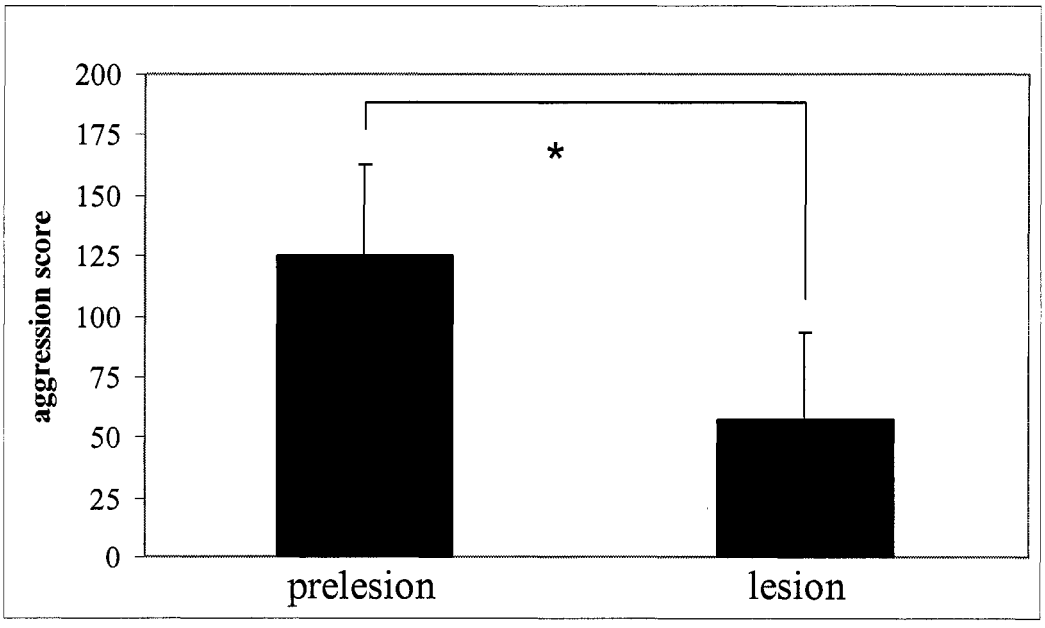

Figure 4.3. Mean (+SE) aggression score in response to stranger song before and after the lesion surgery.

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