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Emerging Auditory Selectivity in the Caudomedial Neostriatum of the Zebra Finch Songbird

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NEOSTRIATUM OF THE ZEBRA FINCH SONGBIRD**

Caroline Wan-Yin Ang

A thesis presented to the faculty of
The Rockefeller University
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

New York, 2001



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To my parents

Acknowledgement

It has been a privilege these last six years to have the opportunity to work with so many talented and creative people. Without them, this thesis would not have been possible.

I would first like to thank Fernando Nottebohm for allowing me the freedom to pursue my own course of research, and in whose lab this work was done. I owe a huge debt of gratitude to everyone in the lab, most notably David Vicario, Constance Scharff, and Claudio Mello for their expert scientific and technical advice. The expertise and generosity of Sharon Sepe, Daun Jackson and Helen Ecklund ensured I was never in need of test subjects. To Sidarta Ribeiro and Guillermo Cecchi, I am eternally grateful for their friendship, optimism, and always managing to find a needle in the haystack of my data. Special thanks to Marc Schmidt for teaching me chronic electrode recording, acting as my external examiner, and being a great colleague and friend. Mike Chen was an invaluable help while I was setting-up the chronic recording rig. The Rockefeller Instrument Shop was instrumental in building much of the equipment required for my experiments. Thanks to my committee, David Gadsby, and Jonathan Victor, for their generosity and guidance. Pat Griffin in the Faculty Club was always able to provide a safe haven.

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ABBREVIATIONS

BOS	Bird's Own Song
Cb	Cerebellum
DLM	Medial portion of the dorsolateral thalamus
DM	Dorsomedial portion of the intercollicular complex
H	Hyperstriatum
Hp	Hippocampus
HVC	High Vocal Center
IMAN	Lateral magnocellular nucleus of the anterior neostriatum
LPO	Paraolfactory lobe
MLd	Mesencephalicus lateralis, pars dorsalis
mMAN	Medial magnocellular nucleus of the anterior neostriatum
MUA	Multiunit activity
NCM	Caudomedial neostriatum
NXIIIts	Twelfth tracheosyringeal nerve of the hypoglossal nucleus
Ov	Ovoidalis
pHVC	ParaHVC
RA	Nucleus robustus of the archistriatum
ZENK	Acronym for zif-268, egr-1, NGF-1, Krox-24

ABSTRACT

The ability to identify and interpret auditory stimuli from the environmental milieu is of particular importance in species that communicate using learned vocalizations. By chronically recording multiunit neuronal responses in awake, behaving zebra finch songbirds, I have demonstrated that the caudomedial neostriatum (NCM), a telencephalic nucleus in the ascending auditory pathway, exhibits responses that range from unselective to highly selective for species-specific stimuli. Sites demonstrating habituating responses are also found and are arranged heterogeneously with selective sites that do not habituate. During the act of singing, activity changes at the habituating sites becoming nonhabituating with neuronal firing occurring at distinct points in the song. Together, these data suggest the involvement of NCM in the processing of behaviorally significant stimuli both for passive audition and for auditory feedback.

CHAPTER 1: INTRODUCTION

I am specifically interested in understanding where the ability to resolve ethologically-specific auditory stimuli emerges in the brain. The focus of this thesis involves elucidating the function and organization of the zebra finch caudomedial neostriatum, or NCM, a region in the ascending auditory pathway. NCM exhibits selective responses to behaviorally relevant stimuli and may be a primary nucleus in the processing of selective auditory information. NCM may also play a role in the auditory feedback loop required for song maintenance since neuronal activity differs during the act of singing versus passive listening.

On the following pages, I will review the influence of hearing on songbird behavior as it relates to the main theme of the dissertation. I will then provide an overview of songbird auditory processing, leading up to the work that directly precedes the studies described in the thesis.

Oscine songbirds, like humans, rely on learned vocalizations as a form of communication. The zebra finch songbird (*Taeniopygia guttata*) provides a good model system for studying complex behavior based on a form of vocal interaction. Although only male finches sing, both males and females must listen and be able to distinguish among the various unique songs as a means of identifying mates, group members, neighbours and strangers (Kroodsma and Byers, 1991; reviewed in Marler and Doupe, 2000). In addition, auditory information is necessary for song learning and maintenance, mate selection and communication (Marler and Peters, 1982; Searcy et al., 1981; Godard, 1991; Brooks and Fall, 1975; Godard, 1991). Much is known about the areas of the brain involved in the motor production of learned song. Less understood are the pathways that analyze incoming auditory information.

The Role of Hearing in the Acquisition and Maintenance of Song

The auditory system plays an integral role in song learning in juvenile birds and song maintenance in adults. Song is orally passed down from one generation to the next. Male finches are

taught how to sing by an adult male tutor. Though the tutor is usually the father, social bonds are not necessary since learning also occurs when young birds hear song that is played from a speaker (Marler and Tamura, 1964; Tchernichovski et al., 1999). Song development begins in the juvenile stage around day 25 post-hatch, until sexual maturity by day 80 (reviewed by Nottebohm, 1993). Young birds first enter a sensory learning period during which time they listen to and memorize a copy of the tutor's song (Marler and Peters, 1982). The memorized copy becomes a template upon which the future song will be based. The sensorimotor period which follows, around day 50, begins with the first attempts at vocalization and song imitation. Vocal output continues to mature as more song-like elements appear during the plastic song stage. During this period, individuals are comparing vocal output with the stored template. If auditory feedback is disrupted by deafening during the sensory, or sensorimotor stage, birds fail to develop normal song (Konishi, 1965). By day 80, a complete and stable song has crystallized that remains largely unchanged for the remainder of the bird's life span (approximately 2-5 years). Each individual's song is unique, and is not a direct

copy of the tutor's song. The accuracy in imitating the tutor's song is dependent on the fidelity of the memorized template, though new song is often subject to a certain degree of improvisation during its development. While a large part of song acquisition involves learning, some features do appear to be innate. White-crowned sparrows raised and taught song in isolation learned only the songs of white-crowned sparrows and not those of other species when presented under similar conditions (Marler and Tamura, 1964). However, this phenomenon may be the result more of physiology than inborn preference since birds from one species may have difficulty imitating the sounds of another species due to the physical make-up of their song production apparatus.

Upon reaching sexual maturity, crystallized song was largely believed to be independent of auditory feedback since deafened mature birds maintained their song for much longer than juveniles (i.e. weeks versus days; Konishi, 1965; Bottjer and Arnold, 1984). However, a subsequent study by Nordeen and Nordeen (1992) demonstrated that song deteriorated in deafened adult zebra finches after about 16 weeks. Lombardino and Nottebohm (2000) have subsequently shown that song production becomes less reliant

on auditory feedback as the bird ages since deafening is most effective at disrupting song in birds just after song crystallization and becomes less potent in older birds. Other groups temporarily manipulated auditory feedback by removing the cochlear hair cells in bengalese finches and allowing them to regenerate (Woolley and Rubel, 1997), or by playing zebra finches altered versions of their song through a loudspeaker during singing (Leonardo and Konishi, 1999). In both cases, the bird's own song (BOS) changed markedly, but then returned to the original form once normal hearing was restored. Both reports interpreted the recovery of song as suggesting the long-term presence of a stored BOS template. Alternatively, or in addition, the changes may have also been due to short-term adaptations by the song motor system to the altered auditory information.

Audition and Behavior

The processing of sounds independent of the bird's own vocal output is also necessary for an individual's survival in its native environment. Male zebra finches, like many songbird species,

remember the songs of familiar neighbouring birds as a strategy for identifying interlopers who may want to challenge for territory and/or mates (Godard, 1991). In the field, it was shown that white-throated sparrows (another songbird species) counter sang strongly in response to playbacks of song from a stranger, compared to song from a neighboring bird (Brooks and Falls, 1975). Male birds are better than females at song discrimination. An operant conditioning study done by Cynx and Nottebohm (1992) observed that male birds were most quickly able to select their own song (Bird's Own Song, or BOS) from songs of other birds in their own aviary. Songs from the bird's own aviary were remembered more easily than songs not heard before. In contrast, females could not discriminate between songs from their own aviary and novel songs. In addition to suggesting gender differences in auditory processing, the work also indicates that males are better able to recognize their own song, even when it originates from an outside source.

Despite evident gender differences in song discrimination abilities, auditory responsiveness is also important for females during mate selection. Male zebra finches use song to court female birds (Searcy et al., 1981). The complexity and length of a song is

thought to be representative of a bird's overall fitness (Kroodsmas and Byers, 1991). Females may use song both to assess potential mates for suitability, and to remember and identify individual birds.

Auditory discrimination and function apply not just within species (conspecific). In song discrimination tasks comparing conspecific with heterospecific (other species) or synthetic song, birds consistently chose to respond to their own species, either by vocal response, in the case of males (Peters et al., 1980), or by copulation-solicitation displays for females (Searcy et al., 1981). Birds appeared to use the temporal patterns and syllabic features unique to their own species' song as criteria for preferential response.

Thus, the ability to select conspecific sounds prevents the auditory system from becoming overwhelmed by irrelevant auditory information which might otherwise result in inappropriate behavior by the individual.

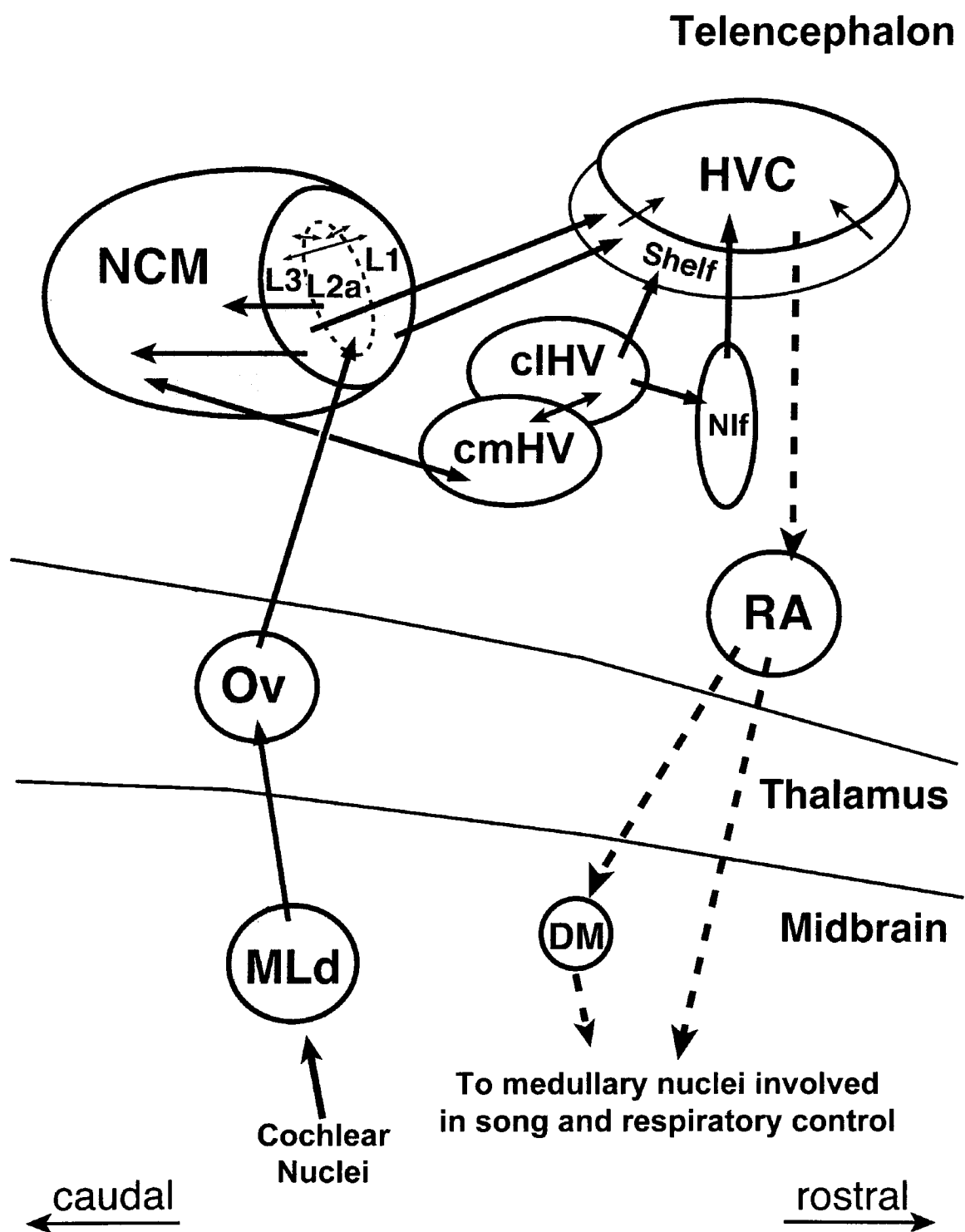
Anatomy of the Auditory Pathway

The primary auditory pathway in the avian brain follows an ascending series of anatomically distinct relays. As summarized in figure 1 (blue areas), sound first reaches the cochlear nuclei, then travels to the nucleus mesencephalicus lateralis pars Dorsalis in the midbrain (MLd; equivalent to the inferior colliculus in mammals; Karten, 1967). Sound information is subsequently passed to the thalamic nucleus ovoidalis (Ov; homologue of the medial geniculate in mammals; Karten, 1968). Ovoidalis sends inputs to the telencephalon, innervating Field L, a large area that is especially prominent in songbirds (Langner et al., 1979; Scheich et al., 1979; Fortune and Margoliash, 1992; Vates et al., 1996).

Field L is the primary auditory relay of the forebrain for projections arriving from Ov (Bonke et al., 1979; Vates et al., 1996) and has been proposed to be analogous to the supragranular layers of the mammalian auditory cortex (Mello et al., 1998b). In anatomical studies, five subregions have been identified: L1, L2a, L2b, L3, and L (Fortune and Margoliash, 1992). These subregions form a web of connections both with each other and with areas lying upstream. Field L2a receives the bulk of inputs from Ov and

Figure 1. A schematic diagram of the ascending auditory pathway and its relation to the song-motor pathway.

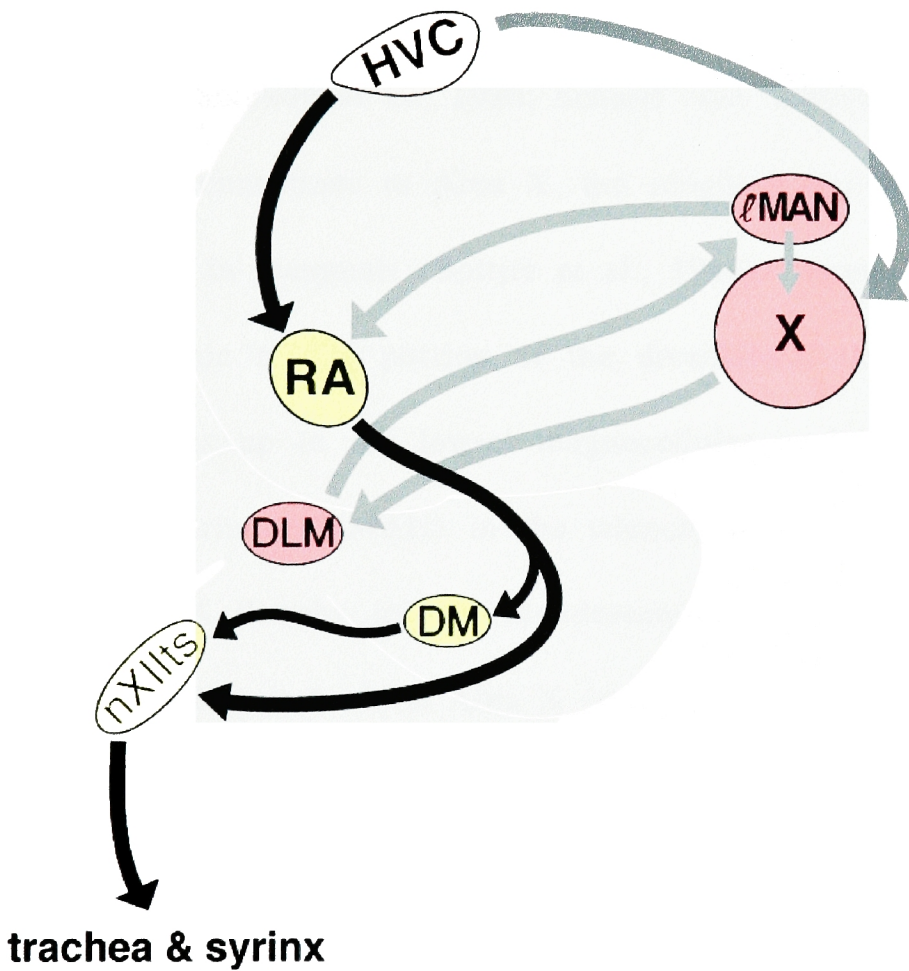
The auditory pathway is shown in blue, and the descending song motor pathway is highlighted in yellow. Sound first enters the ear passing through the cochlear nuclei to the mesencephalicus lateralis pars Dorsalis (MLd; equivalent to the inferior colliculus in mammals), then to the thalamic structure ovoidalis (Ov; the medial geniculate in mammals) which inputs Field L2a. L2a is a subregion of a large area generally referred to as Field L. L2a interconnects with Fields L1 and L3. Both L2a and L3 input to NCM, L2a to the rostral extent of NCM and L3 throughout. NCM is interconnected with cmHV, which in turn interconnects with its more lateral extent (clHV). clHV sends inputs to structures (Nif-nucleus interfaccialis and HVC shelf) that ultimately synapse onto the High Vocal Center (HVC), one of the major regions in the song motor pathway. L1 and L3 also input to the HVC shelf. The signal to sing probably originates in HVC, and is then relayed to the robust nucleus of the archistriatum (RA). RA sends both direct and indirect (via DM, the dorsomedial nucleus of the intercollicular complex) projections to the midbrain structures involved in the physical production of song. Adapted from a diagram provided by C. Mello.



interconnects with Fields L1 and L3, which are also interconnected with each other. Both L2a and L3 send inputs to NCM; L2a being limited to the more rostral region, while L3 innervates throughout (Vates et al., 1996). NCM is interconnected with cmHV, which in turn interconnects with its more lateral extent clHV. clHV sends inputs to the nucleus interfacialis (Nif) and HVC shelf, structures that synapse onto the High Vocal Center (HVC), a region instrumental in song production. L1 and L3 also project to the shelf providing a more direct input from the auditory pathway to the song system (Kelley and Nottebohm, 1979; Saini and Leppelsack, 1981). The signal responsible for the motor production of song is believed to originate from HVC and follows what is known as the descending motor pathway (figures 1 and 2, yellow regions) that inputs to the nucleus robustus of the archistriatum (RA). RA then sends both direct and indirect (via DM-the dorsomedial nucleus of the intercollicular complex) connections to the tracheosyringeal part of the hypoglossal nucleus (nXIIIts) in the hindbrain and to other brainstem nuclei involved with control of the syrinx the organ directly responsible for song production. Lesioning of HVC, RA or nXIIIts results in gross song deficits

Figure 2. A schematic diagram of the descending motor pathway and anterior forebrain pathway of the song system.

The song system consists of two pathways the descending motor pathway, depicted in yellow, and the anterior forebrain pathway shown in fuchsia. The descending motor circuit is necessary for normal production of song throughout life, while the anterior forebrain pathway is needed during juvenile song learning. Inputs from auditory regions enter HVC which sends connections to both pathways. For the descending motor pathway, HVC inputs to RA, which sends both direct and indirect (via DM) connections to nXIIts - the nerve that directly controls the muscles of the syringeal organ. HVC also projects to Area X of the anterior forebrain circuit which inputs DLM, which in turn sends connections to IMAN. IMAN projects both to RA of the descending motor pathway, and back to Area X resulting in an anterior forebrain feedback loop.



(Nottebohm et al., 1976; Nottebohm et al., 1982; Vicario and Nottebohm, 1988).

The ascending auditory pathway may also integrate with the anterior forebrain pathway (figure 2, fuchsia regions), the circuit responsible for song learning and production in juveniles (Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991). HVC sends connections to Area X, the songbird equivalent of the basal ganglia in mammals (Bottjer et al., 1989). Area X then inputs to the thalamic medial portion of the dorsolateral thalamus (DLM), which projects up to the lateral magnocellular nucleus of the anterior neostriatum (lMAN) in the telencephalon. lMAN cells project to RA thereby joining the descending song motor pathway. lMAN also projects back to Area X resulting in the formation of a feedback loop within the anterior forebrain pathway (Vates and Nottebohm, 1995). Lesions in these nuclei cause marked disruptions in juvenile song, but have no obvious effect on stable crystallized song.

ParaHVC (pHVC), a structure recently identified by Foster and Bottjer (1998) extends from the medial region of HVC and receives inputs from HVC, parts of mMAN and Nif. It projects to area X, and

reportedly to the most dorsocaudal extreme of NCM. This area potentially provides a novel projection from the song pathway to the auditory pathway and suggests the possibility of a feedback loop from the song circuit back to the auditory system.

Auditory Selectivity in the Brain

Previous work studying the nature of neuronal activity in the ascending auditory pathway has focused largely on the regions up to and including Field L. DM, MLd, and Field L are all tonotopically organized (Leppelsack and Vogt, 1976; Müller and Scheich, 1988; Theurich et al., 1984a and b). In the case of Field L2, tonotopy is maintained in part by GABAergic inhibition since treatment with a GABAergic antagonist results in an expansion of isointensity response areas (Müller and Scheich, 1988). Some specificity to short-duration species-specific sounds has been observed to emerge in auditory regions up to and including Field L. Leppelsack and Vogt (1976) found cells in the Field L of starlings that responded preferentially to species sounds. Lim and Kim (1997) were able to show similar preferences to zebra finch-like harmonic complexes in Fields L1 and L3, and lack of differentiation in L2. Similarly, in the

nonsinging guinea fowl, Langner et al., (1979) have shown that Fields L1 and L3 are both selective to species calls compared to synthetic stimuli, while Field L2 cannot discriminate. These studies demonstrate that Field L, or at least subregions L1 and L3 are capable of stimulus discrimination. However, all the stimuli used were short-lasting, for example bird calls, or pairs of syllables excerpted from song. When more complex whole song stimuli were tested, none of the Field L subregions discriminated between normal and altered song (Lewicki and Arthur, 1996). Margoliash (1986) has shown that rates of activity to playbacks of BOS and conspecific song are indistinguishable in Field L. Activity also does not differ when birds are singing compared to when listening to BOS (M. Schmidt, personal communication). Thus, while subregions of Field L can discriminate short duration species-specific sounds, this nucleus appears to be an unselective auditory relay for complex external and self-generated vocalizations.

Investigations into other regions of the songbird brain that might give rise to auditory selectivity then turned to the descending motor, and anterior forebrain pathways of the song system. An astonishing preference for BOS compared to reverse

BOS and conspecific song is exhibited in HVC when recording from anesthetized animals (Katz and Gurney, 1981; McCasland and Konishi, 1981; Margoliash, 1986). Auditory activity in HVC is difficult to detect in awake birds and becomes robust only when subjects are anesthetized or asleep (Schmidt and Konishi, 1998; Dave et al., 1998). BOS-specific neurons respond synchronously throughout HVC (Sutter and Margoliash, 1994), and extracellular, single-unit activity is either tonic (i.e. responding strongly throughout playback), or phasic (i.e. responding at specific points in the song; Lewicki, 1996). Neurons are sensitive to temporal order and harmonic (frequency) characteristics unique to BOS (Margoliash and Fortune, 1992; Lewicki and Konishi, 1995; Lewicki and Arthur, 1996). This response emerges in parallel with song development. Volman (1993) demonstrated by extracellular recordings in anesthetized animals that juvenile songbirds in the plastic song stage preferred forward tutor song and bird's own subspecies song to that of reverse, or conspecific song. In nonsinging tutored birds, neurons responded equally well to forward and reverse tutor song. Recordings in the HVC and HVC shelf of white-crowned sparrows in the sensory learning phase (i.e.

pre-singing) revealed that HVC selects against heterospecific stimuli compared to normal, reverse and modified conspecific song, while the shelf responded equally to all stimuli. During singing, auditory responses are not present in HVC since neuronal patterns remain the same after deafening (McCasland and Konishi, 1981). Thus, at the level of HVC, auditory information is reaching the song system at impoverished levels in the awake, behaving animal, and auditory feedback does not occur in HVC during vocal production.

Auditory responses similar to those in HVC were also demonstrated in other parts of the song system. Recordings in RA found preferential responses to BOS (Vicario and Yohay, 1993). Lesions in HVC abolished auditory activity in RA, while IMAN lesions had no effect suggesting RA receives auditory information from HVC and not via the anterior forebrain pathway. BOS-selective activity is also found in IMAN, and area X, though some responses in area X and DLM do not discriminate between forward and reverse BOS (Doupe and Konishi, 1991). Preferential tuning to BOS and tutor song in IMAN is found in juvenile birds (Solis and Doupe, 1997). Presumably, BOS preference emerges as the song stabilizes, as in HVC. Area X neurons do not appear to be as finely

tuned as in IMAN since activity in this nucleus could not discriminate between BOS and conspecific song, but could against reverse BOS. During singing, auditory input was found not to contribute to activity in IMAN and area X (Hessler and Doupe, 1999b). These data support the report by Jarvis and Nottebohm (1997) that the expression of a molecular marker for neuronal activity was induced by singing in HVC, IMAN and area X of birds independent of hearing. Thus, like the studies done in HVC and RA of the descending motor pathway, IMAN and area X of the anterior forebrain circuit are sensitive to BOS playback under certain behavioral conditions, but do not appear to be involved in auditory feedback during singing.

Auditory Selectivity in NCM

Many of these studies made the assumption that the auditory processing of ethologically relevant information, particularly as it relates to BOS, occurs in nuclei devoted to the production of song. Because of the pattern of responses observed in Field L, the ascending auditory pathway was regarded as an indiscriminating

circuit supplying inputs to the song regions. However, auditory feedback is not observed in the song nuclei during singing (McCasland and Konishi, 1982; Hessler and Doupe, 1999). As well, stimulus playback elicits robust responses to BOS in the song nuclei of asleep or anesthetized animals. In contrast, awake behaving subjects exhibit inconsistent and/or inhibited activity (Katz and Gurney, 1981; McCasland and Konishi, 1981; Margoliash, 1986; Schmidt and Konishi, 1998; Dave et al., 1998; Vicario and Yohay, 1993; Doupe and Konishi, 1991). Together, these data suggest that the transformation of sound information must be taking place somewhere other than Field L and the song regions.

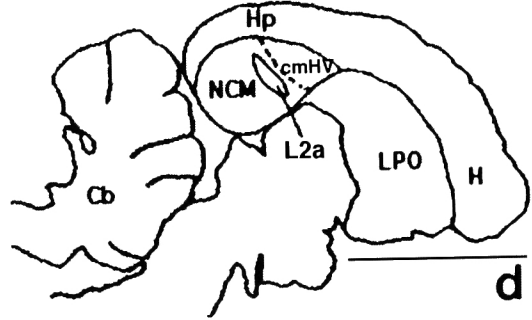
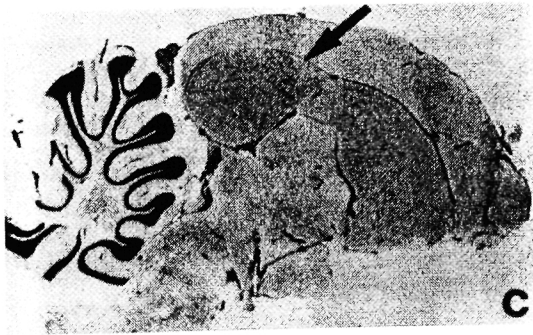
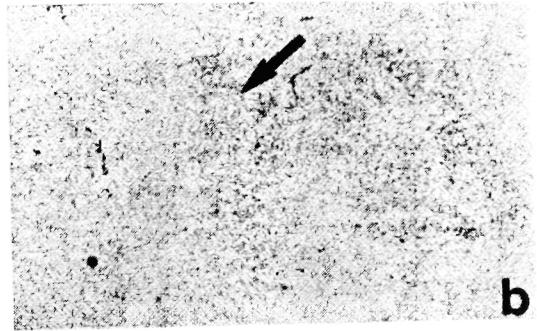
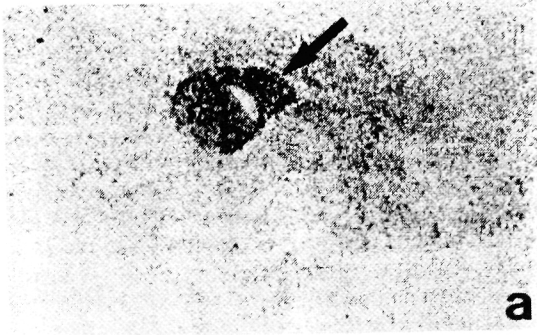
To identify new brain regions involved in the processing of auditory information, molecular studies carried out by Mello et al., (1992) employed the use of the immediate early gene ZENK (an acronym for zif-268, egr-1, NGFI-A, and Krox-24) as a marker for neurons undergoing activation. ZENK is a zinc-finger transcriptional regulator that is induced in association with neuronal depolarization (Goelet et al., 1986; Chaudhuri, 1997). Mimicking the natural behavior, conspecific song was played to birds in acoustic isolation for 30-40 minutes from a speaker. Birds

were immediately sacrificed, their brains sectioned and tested for ZENK expression by *in situ* hybridization. Selective expression of ZENK mRNA was detected in NCM (figure 3), a region in the ascending auditory pathway described above (figure 1). ZENK expression was also found in cmHV, and to a weaker degree in Fields L1 and L3, and the HVC shelf (Mello and Clayton, 1994). The lack of expression in the primary auditory relay (i.e. MLd, Ov, and field L2a) was surprising and suggested that the activation detected by ZENK upregulation was more than just the result of auditory stimulation. Indeed, studies found that the expression of ZENK in NCM was best induced by conspecific song compared to heterospecific song (i.e. canary) and pure tones (Mello, et al, 1992). As well, a recent study by Ribeiro et al., (1998) has shown that the presentation of song syllables induces a pattern of ZENK protein expression in the NCM of canaries that is different from the pattern produced by artificial stimuli sharing the same frequency characteristics. These expression maps are not simple combinations that would result from the activation of a classical tonotopic map (Müller and Leppelsack, 1985), but appear to represent a pattern of cellular response specific to species song.

Figure 3. Expression of ZENK mRNA in the brain after song playback

Parasagittal sections corresponding to the 250 μ m lateral from the median. Dorsal is up and rostral is to the right. a) *In situ* hybridization to ZENK mRNA in an adult male zebra finch exposed to conspecific song playback for 45 minutes. b) A silent control. c) Cresyl violet staining of the section shown in (a). d) A camera lucida rendering of the histological section illustrated in (c). The arrows point to the site of high levels of ZENK induction that encompasses NCM and the caudomedial hyperstriatum ventrale (cmHV). Cb, cerebellum; H, hyperstriatum; Hp, hippocampus; LPO, paraolfactory lobe; L2a, Field L2a (Bar = 4 mm)

From Mello et al., 1992.



The timing of ZENK expression is typical of other immediate-early genes, i.e. mRNA levels peak around 30 minutes from the start of stimulus presentation, then decline to near background levels by 60 minutes. Continued presentation of the original stimulus fails to maintain expression of the gene. However, presentation of a novel stimulus again results in strong expression of ZENK in NCM, though whether this reinduction occurs in the same cells, or new ones cannot be determined from molecular studies (Mello et al., 1995). Further suggestive evidence for the role of NCM in remembering song was provided by Jarvis et al., (1995) who demonstrated that song paired with a mild shock was better at inducing ZENK mRNA than playing novel song. ZENK expression behavior in NCM is similar for both hemispheres and in both male and female birds. During the act of singing, ZENK mRNA is upregulated in NCM, Fields L1 and L3, cmHV and HVC shelf, in addition to the song regions described above (Jarvis and Nottebohm, 1997; Mello and Ribeiro, 1998). Unlike the song nuclei, expression in auditory regions disappears upon deafening indicating molecular activity is induced by auditory feedback and not motor activity. Expression remains sustained in NCM during singing, though at lower levels than

initially observed for playbacks of novel song. NCM may also be tuned to BOS since ZENK mRNA induction following playback of tutor song is higher in adult birds who sing good copies of the tutor song compared to birds with weaker copies (Bolhuis, et al., 2000).

Subsequent electrophysiological studies in NCM demonstrated that playing conspecific song to awake restrained birds caused a decrease, or habituation in activity over time to repeated presentation of the same song (figure 4; Chew et al., 1995).

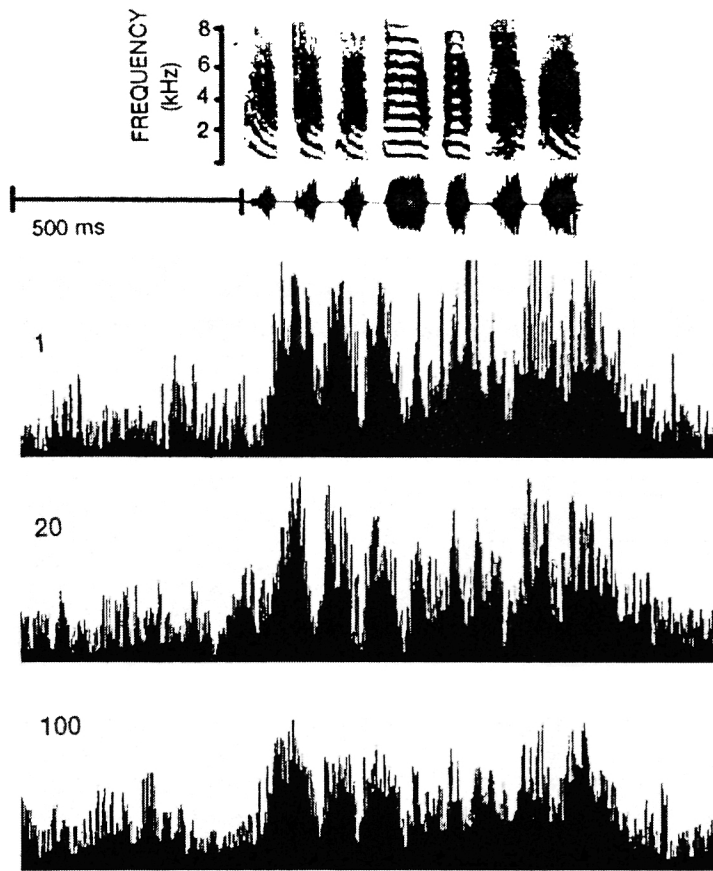
Introduction of a novel song resulted in renewed levels of activity followed again by habituation. Again, activity was present in both male and female birds and did not differ between the left and right side. Habituation to a particular song is maintained for up to 48 hours, and tests to determine the capacity for distinguishing between novel and familiar song have utilized up to 16 songs, although the capacity may be larger (Chew et al., 1996a and b).

Based on the molecular and physiological studies, NCM looks to be a region that exhibits differential activity to learned and novel stimuli. It selects for species and individual-specific auditory information and is sound-activated during singing.

Figure 4. Decrease in multiunit response to repeated presentations of a conspecific song

Diagrams from top to bottom show the sonogram of the novel conspecific song played, the amplitude envelope of the song, and the rectified multiunit response on the 1st , 20th, and 100th song presentation. Activity habituated with an increasing number of trials. Song was presented every 11 seconds to an awake, restrained adult male zebra finch.

From Chew et al., 1995.



A more complete understanding of the function of NCM has been complicated by the fact that NCM is both functionally and anatomically heterogeneous. Not all cells express ZENK at any one time (Mello et al., 1992) and not all sites show habituation, particularly in the more rostral regions (Chew et al., 1995). We know also that NCM receives inputs from auditory areas (specifically, Fields L2a and L3) that map to different parts of the nucleus (Vates et al., 1996).

Taken together, many unanswered questions remain. Among them: How is auditory information assessed with respect to specificity in NCM? Is there a functional organization in the region? Does NCM play a role in auditory feedback? I have attempted to address these issues in the experiments outlined and discussed on the following pages.

CHAPTER 2: Selective Auditory Responses in NCM to Species-Specific Stimuli

Evidence from molecular and physiological studies looking for neuronal regions capable of detecting behaviorally significant stimuli revealed the telencephalic caudomedial neostriatum, or NCM to be involved in auditory discrimination (Mello et al., 1992, 1994, 1995; Chew et al., 1995, 1996a, 1996b; Stripling et al., 1997). Cells in NCM exhibit selectivity for novelty and conspecific song preference, while a major input to NCM, Field L2a, shows no such selectivity. However, further characterization of the function of NCM has been complicated by the heterogeneity of NCM with respect to anatomy and gene expression (Vates et al., 1996; Mello et al., 1992). Thus, I investigated how auditory information was represented throughout the region by 1) classifying functional responses within NCM to sets of auditory stimuli and 2) mapping these responses to determine if NCM is composed of a specific organization.

In order to study neuronal responses in the awake, behaving animal, multiunit recordings were done in chronically implanted,

awake and freely moving animals. Adult male birds listened to bird's own song (BOS), reverse BOS, conspecific song, and heterospecific (canary) song. Multiunit responses in NCM ranged from those that showed little selectivity (similar to Field L2a), to those that were very selective and capable of resolving zebra finch song from canary song, and reverse song. A subset of sites, rather than responding repeatedly to acoustic cues, habituated during stimulus presentation by responding only to the early trials, and exhibited preferential activation in response to the initial portions of the stimulus. Some of these habituating sites also exhibited stimulus selectivity. Further, we have found anatomical correlates for the range in physiological behavior, providing evidence for a functional and anatomical organization of NCM. The physiological complexity of NCM, as well as its position in the auditory pathway, implicates it as a primary station involved in the evaluation of auditory stimuli for ethological importance.

Results

Multiunit recordings were done in awake, freely moving adult male zebra finches (25 sites, 17 birds) fitted with chronically implanted electrodes. We sampled sites across the rostral-caudal extent of NCM. Subjects were played interleaved sets of digitized stimuli (50 presentations of each stimulus) consisting of conspecific zebra finch song, bird's own song (BOS), reverse BOS (rev BOS), and canary song. We chose these four stimuli to evaluate response based on individual specificity (BOS), species-specificity (conspecific song), altered species-specificity (rev BOS), and heterospecificity (canary). For a more detailed description of the stimuli, please see Materials and Methods.

Variations in Stimulus Selectivity

Below, we present responses throughout NCM to the four stimulus types. Responses ranged from robust for all stimuli, to preferentially selective for BOS and conspecific song. In addition, habituation could be present or absent. To illustrate the range in

response, I present four sites that exhibited consistently selective differences in activity. These are as follows:

- a) responded robustly to all stimuli;
- b) responded strongly to BOS, rev BOS and conspecific song, but less vigorously to canary stimulus;
- c) responded robustly to BOS and conspecific song, but not to canary and reverse BOS;
- d) habituated in response to BOS, rev BOS and conspecific song, while showing only weak activity for canary song.

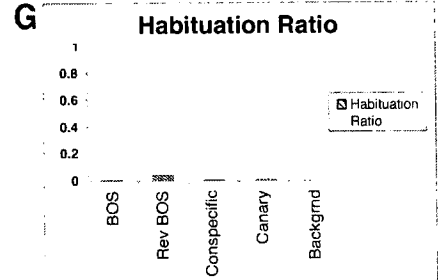
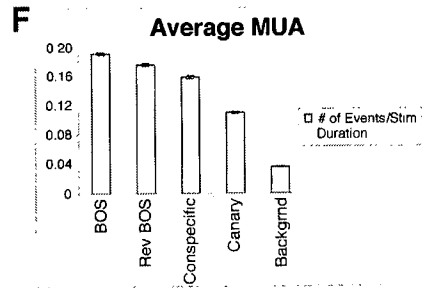
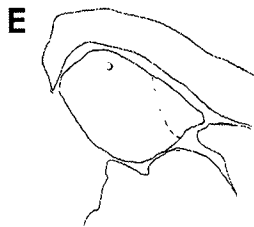
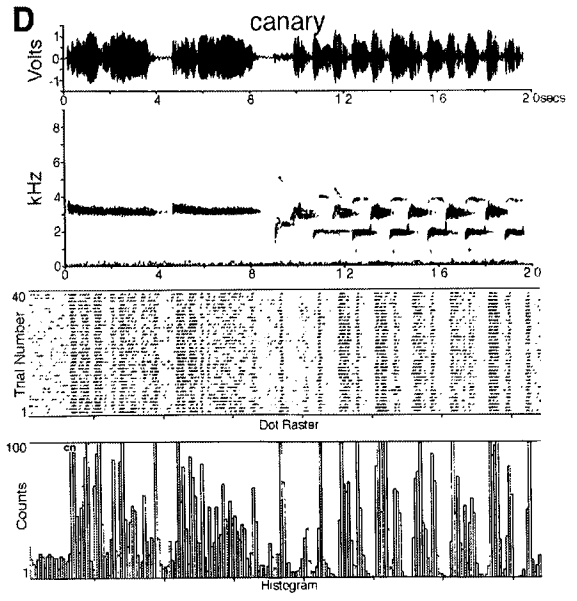
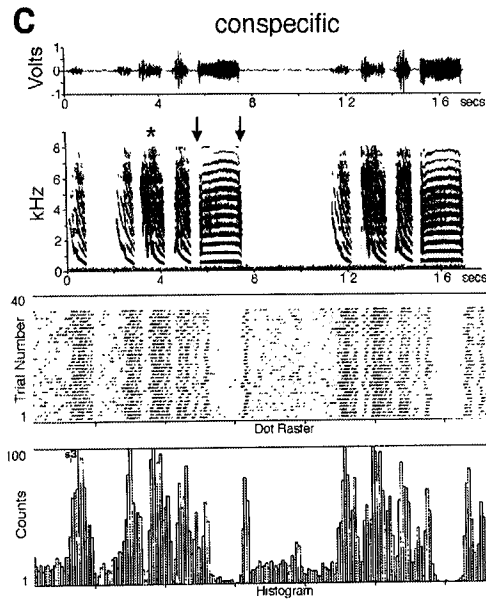
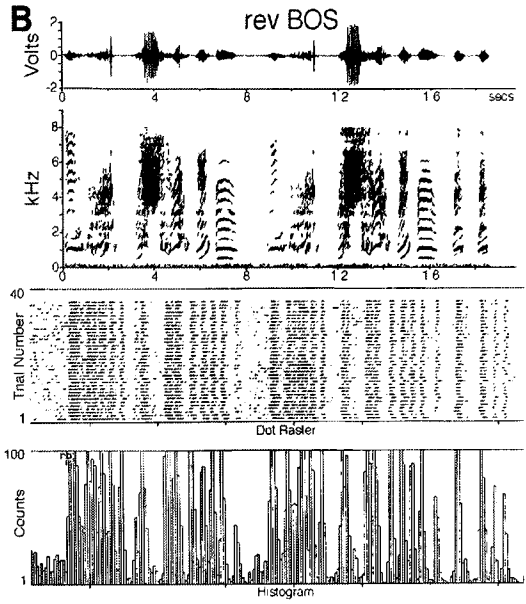
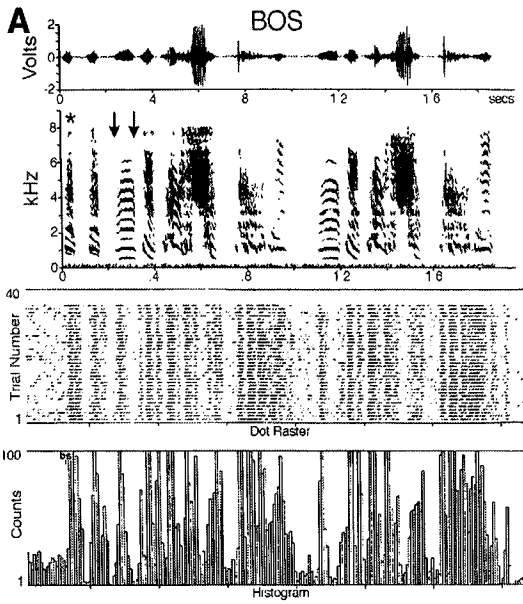
While most recordings showed some degree of decrease in activity over time, sites deemed “habituating” were placed in a separate category, as will be described later.

Sites that Respond to All Stimuli

At one end of the response spectrum were sites that responded robustly to all four types of stimuli tested (6 sites in 5 birds). For each of the four response panels in figure 3, stimulus volume is represented by the amplitude diagram in the uppermost trace. Immediately below, the sonogram separates the stimulus

Figure 5. Site that Responds to All Stimuli

Birds were played stimuli representing A) BOS; B) reverse BOS; C) conspecific song; and D) canary song. Diagrams from top to bottom show the amplitude envelope of the song, song sonogram, dot raster and histogram analysis of activity. All stimuli elicited vigorous and stereotyped responses. Unmodulated, stable stacks elicited sharp on/off responses while activity during the intervening period of the note was inhibited (arrows in A and C). Activity to more modulated syllables occurred for the duration of the note (asterisk in C). E) Camera lucida representation of a parasagittal section showing the location of the recording site in the dorsal extent of rostral NCM. Dorsal is up and rostral to the right. F) The average number of events detected during the stimulus was divided by the stimulus sound duration to give the average multi-unit activity. Responses to all stimuli were high compared to background, though canary showed a modest decrease in activity compared to zebra finch stimuli. G) Activity to all stimuli changed very little over the course of playback as evidenced by the habituation ratio. An habituation ratio close to zero indicates activity that decreased very little, while a value close to 1 indicates a drop in response over the course of the playback. Values normalized to background.



into its component frequencies, with respect to time. Multiunit activity is shown in two forms: dot raster and histogram analysis.

Despite the spectral and temporal differences between canary and zebra finch song, activity was similar to that of the three zebra finch stimuli, i.e. robust and stereotyped, as can be seen in all four dot raster diagrams. Activity occurred throughout each stimulus, but not uniformly to each note. For BOS (figure 5A), rev BOS (figure 5B), and conspecific song (figure 5C) unmodulated stacks of stable amplitude (i.e. loudness) elicited a short-lived phasic response. Sharp on/off responses were observed while activity during the intervening period of the note was inhibited (arrows, figures 5A and C). In contrast, notes containing downsweeps or fluctuations in frequency (asterisks, figures 5A and 5C) induced activity throughout the note's duration. Response to silent periods in the stimulus was similar to spontaneous activity (data not shown). Cells in these sites also appear to be sensitive to amplitude changes, as illustrated by the response to canary stimulus (figure 3D). The first two notes though quite stable in frequency, vary in amplitude, which is reflected in the response.

The average multiunit activity (MUA) was used as an indicator of overall activity for each stimulus and measured the average number of events detected over the course of a presentation (figure 5F). Surprisingly, the qualitative similarities in response between canary and zebra finch stimuli were not reflected in the average MUA. Canary stimulus elicited a modestly reduced response (110 mean events/s \pm 0.001 SE) compared to BOS (191 events/s \pm 0.002), rev BOS (176 events/s \pm 0.002), and conspecific song (158 events/s \pm 0.002). However, all stimuli measured well above background (036 events/s \pm 0.001), specifically by 3.1-5.4 times in excess.

The habituation ratio values were near zero for all stimuli indicating activity dropped very little from the first five trials to the last five trials (figure 5G). The recording site illustrated was located in the dorsomedial region of the middle of NCM (figure 5E).

Thus, responses to the four different stimuli were all robust. Activity was present throughout the stimulus, though there were differences in response to individual notes that appeared to depend on specific acoustic characteristics, including frequency, modulation, and amplitude. As well, a diminution in the response

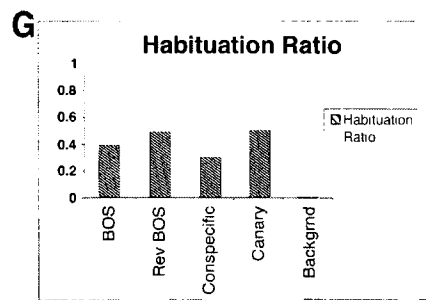
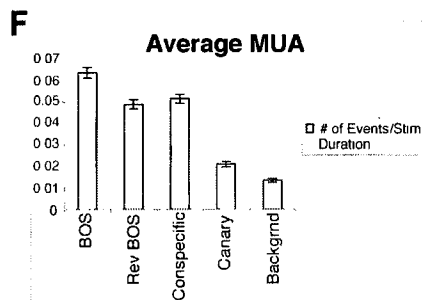
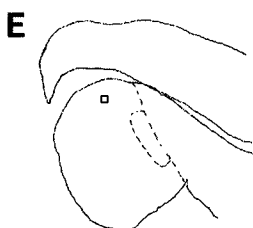
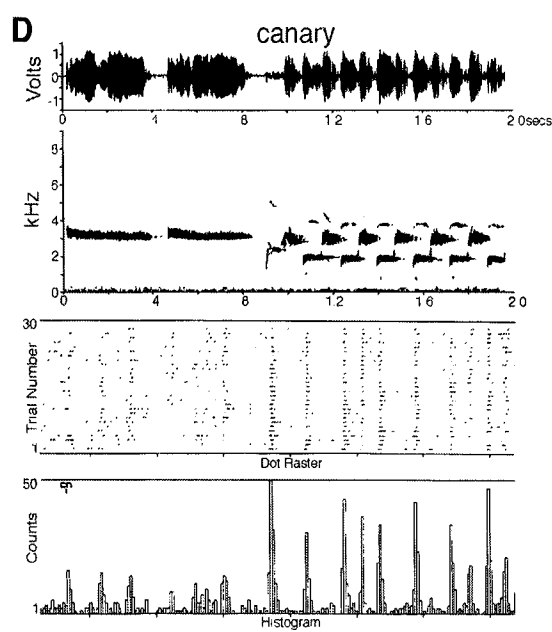
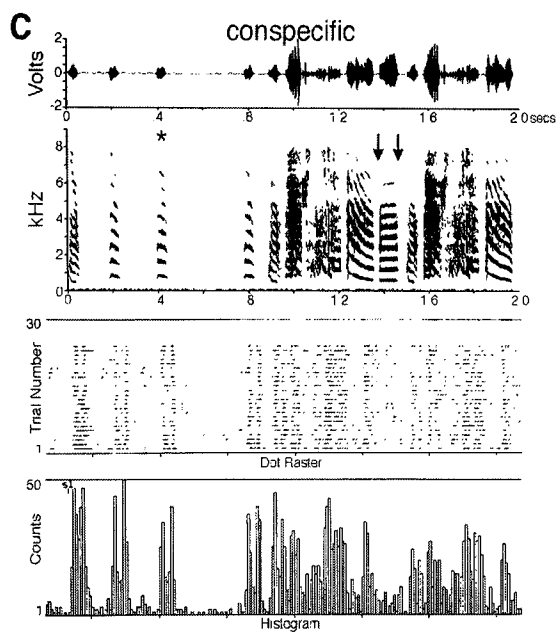
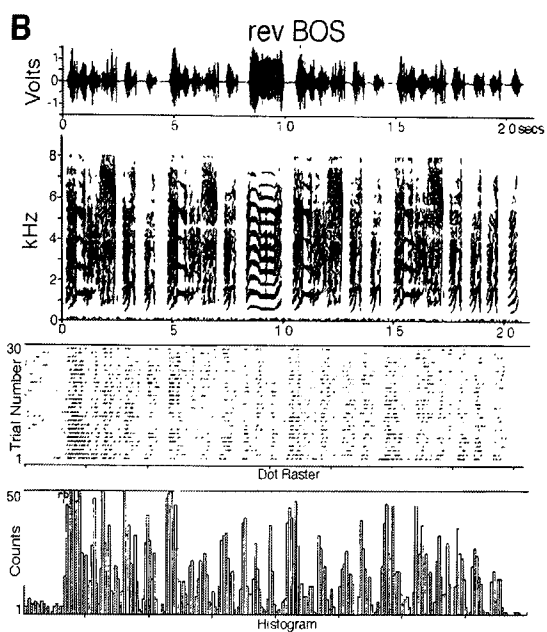
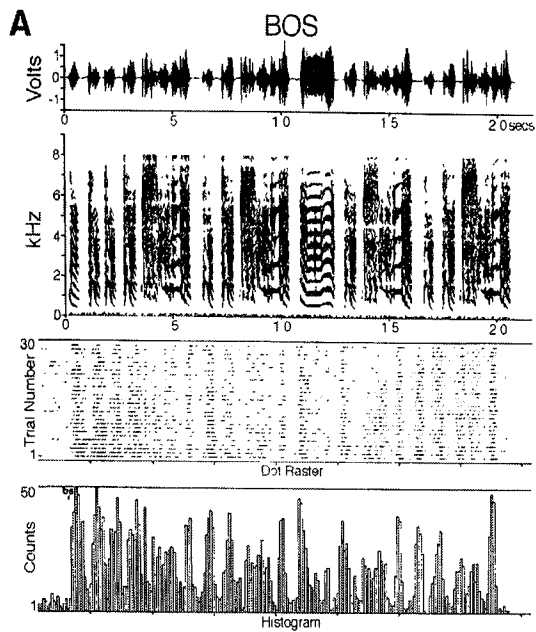
to canary stimulus was observed suggesting some selectivity may be occurring. Such heterospecific selectivity is more dramatically illustrated in the next example.

Sites that Filter Canary Stimulus

Other sites were more intermediate in stimulus preference (7 sites, 5 birds) responding vigorously to BOS (figure 6A), rev BOS (figure 6B), and conspecific (figure 6C), but only weakly to canary song (figure 6D). As seen in figure 6F, the average multiunit activity for canary stimulus was <50% that of values for the three zebra finch stimuli. From the histogram and dot raster diagrams, activity for canary song appears considerably weaker and the response is limited only to specific elements in the latter (trill) portion of the stimulus. Often in sites that filtered canary stimulus, neurons would respond only to specific parts of the canary song. In contrast, activity to the other stimuli occurred throughout (data not shown). There were some differences in the responses to individual notes in BOS, rev BOS and conspecific song in the site highlighted in figure 6 compared to previously mentioned sites.

Figure 6. Site that Filters Canary Stimulus

Diagram organization is as described in Figure 5. Cells responded vigorously to all stimuli except that of canary, which displayed <50% average MUA. On/off activity for unmodulated stacks was no longer observed suggesting modulations in frequency were required to stimulate the cells (arrows in C). Activity was also seen to change from continuous to on/off activity for some notes over the course of playback (asterisk in C). The weak canary response, which contains ample shifts in frequency, indicate that cells in these sites perhaps require additional criteria that specify species relevant sounds. E) The recording site was located in dorsal NCM, caudal to Field L2a (region enclosed by dotted line). Dorsal is up and rostral to the right. F) Average MUA for canary was less than 50% that of BOS, Rev BOS and conspecific song. G) Activity levels decreased by approximately the same amount for all four stimuli over the course of playback. Values normalized to background.



Stable frequency notes did not elicit the sharp on/off activity seen previously (figure 6C, arrows). While responses still appeared to have similar temporal structures, for certain notes as playback progressed from earlier to later trials (from the bottom to top of dot raster, respectively), the spike trace lessened into two distinct lines corresponding to the start and end of the note. This is best illustrated for the note marked by the asterisk in the figure 6C dot raster.

The strong, consistent activity exhibited for BOS (64 events/s \pm 0.002), reverse BOS (49 events/s \pm 0.002) and conspecific song (51 events/s \pm 0.002) was 3.6-4.8 times above background (13 events/s \pm 0.001). Activity in response to the canary stimulus (21 events/s \pm 0.001) was only 1.5 times that of background (figure 6F). Activity decreased the most over the course of playback for rev BOS (0.47 habituation ratio), and canary song (0.47), as indicated by their habituation ratios (figure 6G). Responses to BOS, and conspecific song did not decrease quite as much (0.38 and 0.29, respectively). Despite the decrements in response for all four stimuli over the course of playback, activity patterns were maintained throughout. The recording in this example occurred

dorsal and caudal to Field L2a, in a similar location to the site in figure 5 (figure 6E; Field L2a enclosed by dotted line).

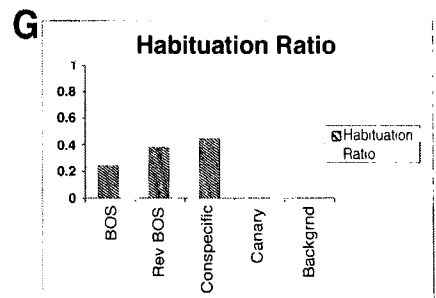
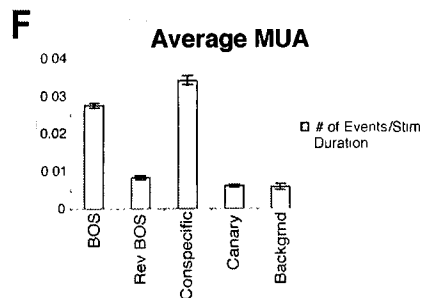
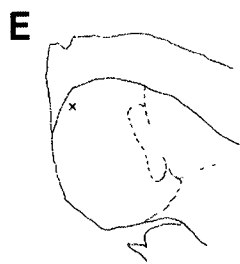
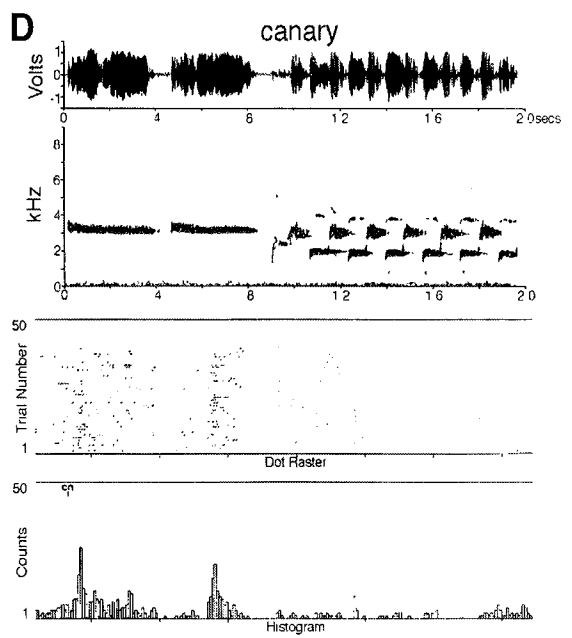
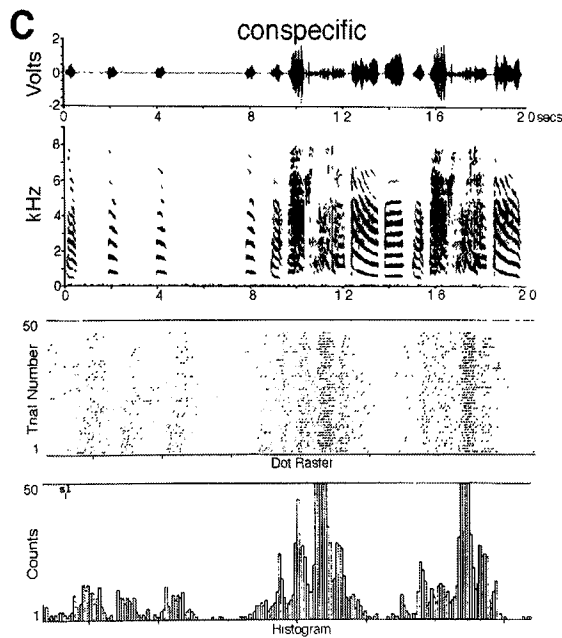
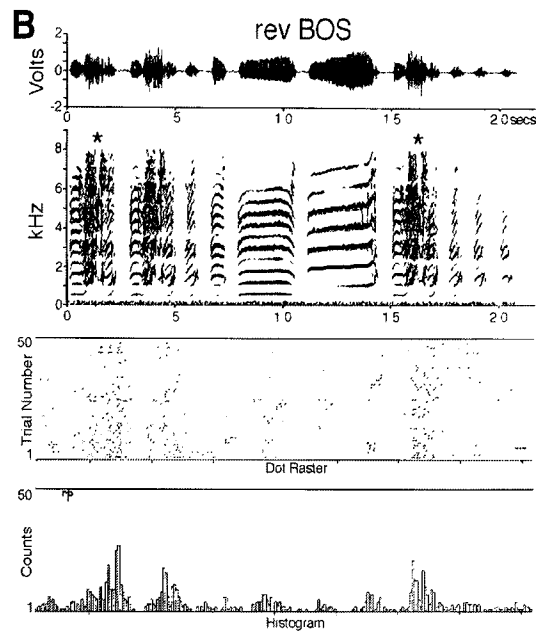
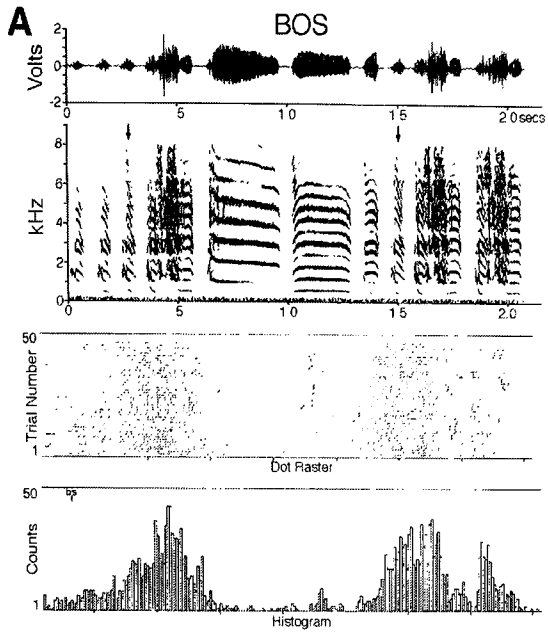
The weak response to the canary stimulus, which contains ample changes in frequency and amplitude, suggests that cells in these sites require additional criteria for activation; criteria that appear to be met by relevant (i.e. species-specific) sounds.

Sites that Filter Canary and Reverse BOS Stimuli

Still higher selectivity was uncovered in NCM within two sites (in 2 birds) that responded strongly and consistently to BOS (figure 7A) and conspecific song (figure 7C), but weakly to rev BOS (figure 7B) and canary song (figure 7D). Activity patterns had a diffuse appearance, as seen in the dot rasters, in contrast with the more sharply defined activity observed in less selective sites. As is best seen in the multiunit activity histograms, activity appeared to be even more restricted in terms of the types of song elements capable of exciting the cells. There was essentially no response to stable stacks, and notes with downsweeping frequencies elicited weak activity. Only harmonically complex, rapidly modulating notes

Figure 7. Site that Filters Canary and Rev BOS stimuli

Diagram organization is as described in Figure 5. Strong activity was recorded for only BOS and conspecific song, while response for rev BOS and canary were both weak. Harmonically complex, frequency modulated notes were the most excitatory, but interestingly, much less so in the case of rev BOS (asterisks in B). E) The recording site was located in the dorsal region of caudal NCM. Field L2a is the enclosed area defined by the dotted line. Dorsal is up and rostral to the right. F) The average MUA for BOS and conspecific was markedly higher compared to rev BOS and canary, which were both close to background. G) Activity for BOS, rev BOS and conspecific song decreased similarly over the course of playback while canary activity changed very little (i.e. started low and stayed low). Values normalized to background.



appeared to be excitatory. Interestingly, these notes played in reverse (asterisks, figure 7B), did not produce as strong a response compared to the forward case. Temporal patterning may also play a role in activation since a syllable in BOS that did not induce much activity when situated as an introductory note elicited much more of a response when embedded in the motif (arrows, figure 7A). This same note produced little, if any activity in the Rev BOS situation (figure 7B). For the canary stimulus, responses were weak, though certain features were sometimes able to produce activity that was sustained throughout playback.

The average MUA shown in figure 7F for BOS ($27 \text{ events/s} \pm 0.001$) and conspecific song ($34 \text{ events/s} \pm 0.001$) were approximately 3.8, and 4.8 times, respectively, that of background ($6 \text{ events/s} \pm 0.001$), while Rev BOS ($8 \text{ events/s} \pm 0.0005$) and canary ($6 \text{ events/s} \pm 0.0003$) were near background. Though the spike counts for rev BOS and canary were both low, they differed in response behavior over the course of the experiment. As indicated by the habituation ratio values (figure 5G), activity for rev BOS declined (0.36 habituation ratio), along with BOS (0.23), and conspecific (0.43), while canary activity (0.00) was weak from the

start suggesting the site may be receiving inputs in which response to canary song has already been filtered. The site was located in the dorsal area of the caudal extreme of NCM (figure 7E).

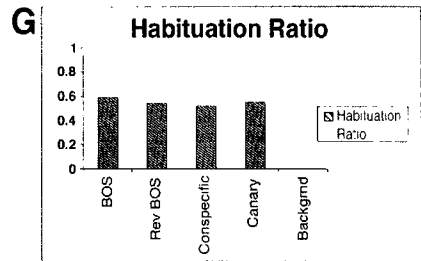
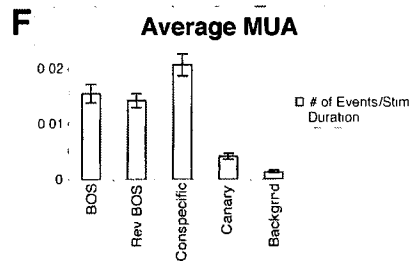
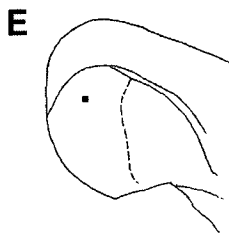
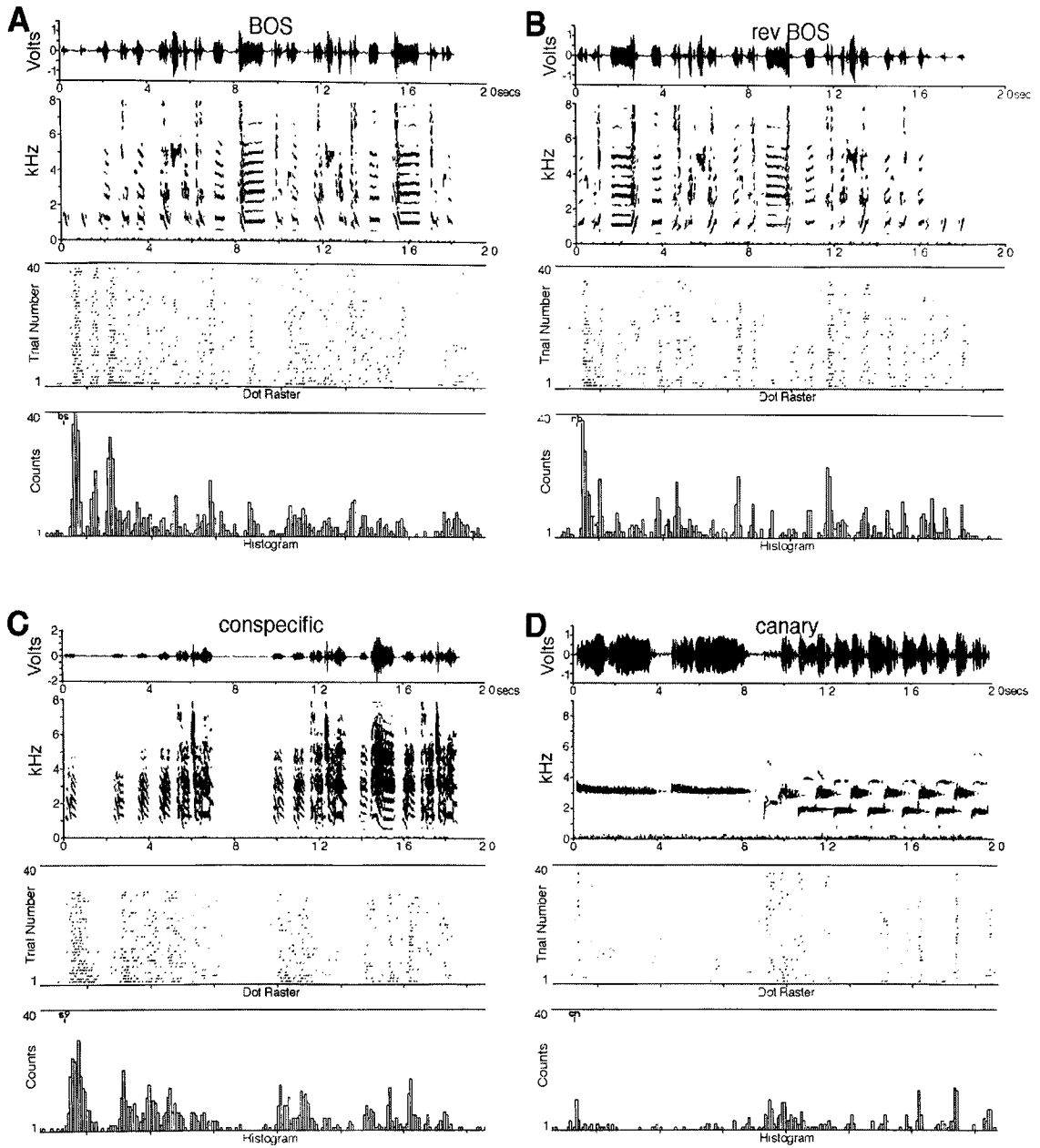
Thus, there are sites in NCM that show relatively high degree of stimulus restriction. Not only do such cells filter out the heterospecific stimulus (perhaps at an earlier processing stage), they also respond only weakly to manipulated (i.e. reverse) zebra finch song. The reasons for these differences are unclear, but may rely on the ability of the cells to recognize frequency modulations and/or temporal characteristics that are specific to normal zebra finch song.

Sites that Habituate and are Transient

An additional type of selective site (10 sites, 6 birds) is shown in figure 8 that, unlike the sites described above, did not necessarily select for specific stimulus characteristics. Instead, activity was strongest for the first several presentations, as has been reported in prior studies to occur in NCM (Chew et al, 1996).

Figure 8. Site that Habituates

Diagram organization is as described in Figure 5. Habituating sites showed vigorous auditory responses to early presentations of novel stimuli followed by decreased activity to subsequent repetitions. Responses were strongest to the initial notes of the stimulus, fading to near background over the course of the presentation. The weak response to canary stimulus was reminiscent of the site highlighted in figure 7. E) The recording site was located in the dorsal area around the center of NCM. Dorsal is up and rostral to the right. F) The average MUA for BOS, rev BOS and conspecific song were markedly higher than for canary and background levels. G) Sites were placed in the habituating category if they possessed a habituation ratio of <50%. Activity generally dropped within the first few stimulus presentations for these sites (see dot rasters). Values normalized to background.



The decrease was not due to a loss of responsive neurons since presenting novel stimuli could reinstate activity (data not shown). Activity was also observed to be most robust for the initial notes of the stimulus, decreasing to near background towards the end of a presentation. Activity that decreased to repeated presentation of a particular stimulus was termed habituating, while activity that decreased during a single response was called transient.

Transience is used here as a general term to refer to the response behavior over the period of an entire stimulus and not to shorter-duration changes that may occur to individual syllables, or small groups of syllables. The example shown in figure 8 is a site that was also selective against canary stimulus (figure 8D), responding most strongly to BOS (figure 8A), rev BOS (figure 8B), and conspecific song (figure 8C). However, not all sites that decreased in activity over playback showed such stimulus filtering.

Habituating activity is best illustrated in the dot raster graphs where activity is observed to be greatest for the earlier presentations. Transient activity can be observed in the histogram graphs where activity at the start of the stimulus is strongest, then weakens in response to subsequent notes. Preferential activity for

the early notes in a stimulus occurs for both BOS and rev BOS, suggesting that temporal order is not a criterion for response.

The average MUA (figure 8F) for canary ($4 \text{ events/s} \pm 0.0005$) measured just above background ($1 \text{ events/s} \pm 0.0003$) compared to BOS ($15 \text{ events/s} \pm 0.002$), rev BOS ($14 \text{ events/s} \pm 0.001$) and conspecific song ($21 \text{ events/s} \pm 0.002$) which were 14-21 times that of background. Sites placed in this category exhibited habituation ratios of >0.50 for all stimuli (figure 8G) and activity rapidly diminished over the first few trials. The site was located in the caudal extreme of NCM (figure 8E).

Thus, for these sites, there is a reduction in activity between successive trials and within an individual trial. These data suggest that the neurons respond preferentially to the initial portion of a stimulus and rely on criteria other than temporal pattern and specific acoustic content for activation. In the case of sites that also exhibit selectivity, responses may reflect pre-filtering from selective cells that exhibit consistent responses trial to trial.

Anatomical Correlates of Physiological Behavior

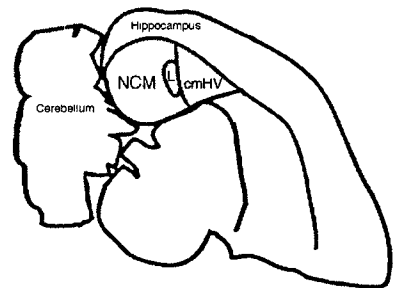
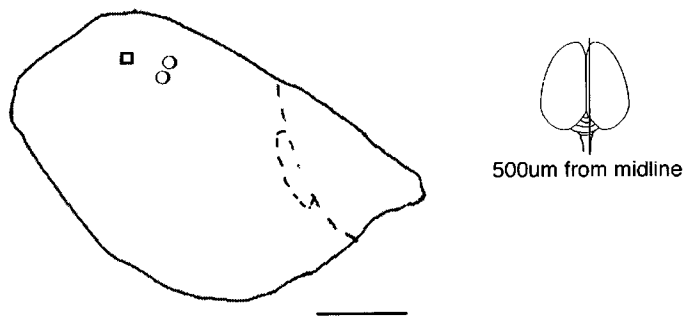
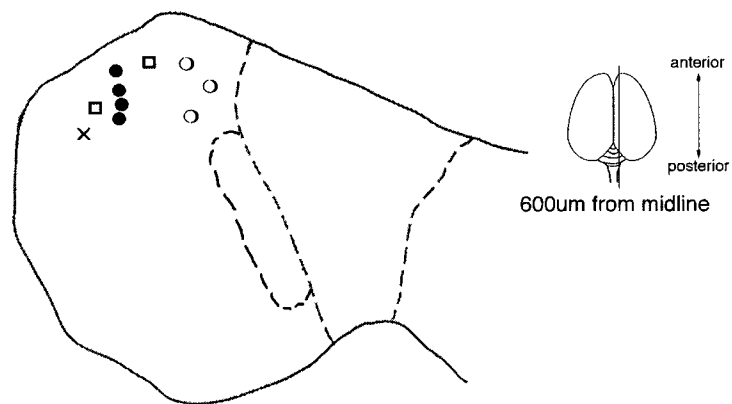
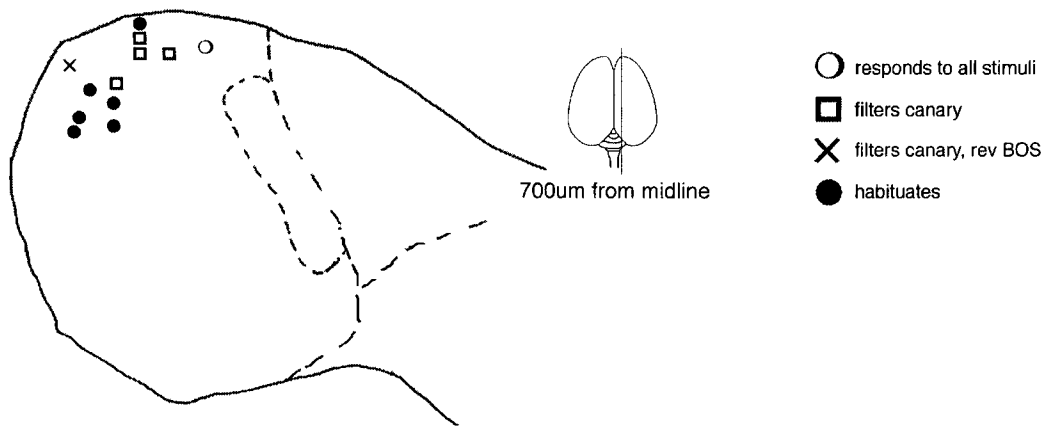
An anatomical map providing the location of the recording sites is necessary to provide a further understanding of the organization of NCM, as well as provide information on how the various stimuli may be encoded in the nucleus. As summarized in figure 9, the locations of the various recording sites are mapped on three parasagittal sections of increasing lateral distance from bottom to top. Sites that responded to all test stimuli were found to lie in the more rostral extent of NCM near the border of Field L2a (open circles, fig 9). Sites exhibiting habituation emerged more caudally, together with song-selective sites that did not habituate. No obvious spatial organization between the different types of selective sites (including those that habituate) was apparent in the caudal region.

Discussion

The influence of the auditory system on behavior is especially significant in animals relying on learned vocalizations for communication. In songbirds, auditory processing of species-

Figure 9. Summary of recording sites and associated activity.

Location of the various sites, both nonhabituating and habituating, in NCM is shown in a series of parasagittal sections moving medial to lateral from bottom to top. The location of NCM and Field L2a in the brain is highlighted in gray in the parasagittal section drawn schematically in the lower right. For all anatomy, dorsal is up, rostral to the right. Sites that responded robustly to all test stimuli are indicated by open circles. These sites were found to lie in the more rostral extent of NCM close to the border of Field L2a. Sites with song-selective filters (open squares and crosses for canary filter and canary/rev BOS filter, respectively) emerged more caudally, along with sites demonstrating habituation. Habituating sites that exhibited vigorous activity to all stimuli are indicated by solid circles, and sites that filtered canary by solid squares. Sites that habituated and selected against canary and rev BOS were not found. No obvious organization between the nonhabituating selective sites and habituating sites was apparent. Scale bar = 500 μ m.



specific information is necessary for acquisition and maintenance of the vocal repertoire, as well as for social interaction. At some point(s) along the auditory pathway, information is transformed from general and unspecific, to highly selective for BOS and conspecific song. The Field L subregion, Field L2a, which sends inputs into rostral NCM is known to respond robustly and with equal strength to most kinds of complex auditory stimuli (Margoliash, 1986; Lewicki and Arthur, 1996; Lim and Kim, 1997). By the time auditory information reaches the high vocal center (HVC), preference for signals representing BOS can be detected. Interestingly, this selectivity seems to be gated and is revealed only in animals that are anesthetized or sleeping (Schmidt and Konishi, 1998; Dave et al., 1998). NCM, which receives auditory information from Field L and projects indirectly to HVC (Vates et al., 1996), has been found in earlier molecular and physiology studies to be a nucleus selective for species-specific song, as well as a detector for novel stimuli (Mello, et al, 1992, 1994, 1995; Chew, et al, 1995, 1996a, 1996b; Stripling et al., 1997). However, the heterogeneous organization of NCM called for further functional and anatomical characterization.

NCM was tested along its rostral to caudal extent in chronically implanted, awake behaving adult male zebra finches. Responses were recorded to sound stimuli that were zebra finch-specific (conspecific, BOS, and rev BOS), and heterospecific (canary song). A variety of activity patterns was observed throughout the region. In rostral NCM, responses were robust to all stimuli. In more caudal regions, a trend towards selective responses emerged. Recordings were obtained from caudal sites that selected against canary song, or canary and rev BOS, while responding vigorously to normal zebra finch BOS and conspecific songs.

Responses to individual notes also varied within NCM. Rostral sites responded with on/off activity to notes of stable frequency and amplitude (arrows, fig 5) and fired continuously throughout harmonically complex notes with modulating frequency and amplitude (asterisks, fig 5). In sites that filtered canary song, cells no longer exhibited on/off responses for stable notes (arrows, fig 6) and activity appeared to lessen and refine over the course of playback (asterisks, fig 6). Neurons that filtered both canary and rev BOS responded exclusively to notes with complex harmonics and frequency shifts, but not in the reverse case (fig 7). Rev

BOS/canary selective sites may be integrating simple input data to produce a complex result. Frequency shifts in zebra finch song are often characterized by downsweeps. The selection against rev BOS may indicate the presence of downsweep detectors at these sites. Field L is known to have tonotopic organization, and might fire in a distinct pattern for notes with frequencies that modulate downwards. Neurons at the selective sites may be sensitive to such a pattern, requiring input from the tonotopic cells to arrive in a particular sequence in order to fire. Upsweeps, or unmodulated notes would not produce the correct firing sequence and therefore would fail to activate.

These three types of sites appear to represent increasingly selective stages of auditory processing that rely upon certain acoustic (i.e. harmonic content, frequency and amplitude modulation) and temporal features characteristic of zebra finch song for activation. However, testing of the syllables alone should also be done to determine if such a response is independent of context, or requires the syllable to be embedded within a specific environment. Whether such responses are hard-wired or experientially determined is not known. Experiments studying

auditory areas involved in song learning suggest NCM may be a region where at least some neurons are tuned during development to respond to elements of tutor song (Bolhuis et al., 2000). In some song nuclei, selectivity of auditory responses is known to emerge in juvenile birds (Solis and Doupe, 1997). Whether this occurs in NCM is not yet known.

Sites that habituated were also detected, sometimes exhibiting stimulus preferences. Responses at these sites began robustly, then faded both over the course of a presentation and between presentations. We have restricted our definition of habituation to only those sites showing strongest activity to the initial notes of the stimulus. The type of note (e.g. introductory note, versus motif note) does not appear to be important, since reverse song still produces the same effect. These sites do not appear to be sensitive to acoustic, or temporal factors. Rather, habituating neurons may be analyzing, or assessing the first notes of a stimulus in order to determine if it is novel or familiar, and, in the case of selective habituating sites, if the stimulus is zebra finch-specific.

Our results provide a wide sampling of responses in NCM. Habituating sites were found to be located in the same region as

sites that selected against canary and rev BOS. Placing these different types of cells in close physical proximity would allow filtered inputs to reach habituating cells more efficiently, and/or vice versa. Indeed, some habituating sites also showed selection. Such neuronal activity is consistent with an interaction between the different types of sites and may account for the variability in responses observed throughout the nucleus. As more recordings are made in this region, a more definitive organization may emerge. For the less selective responses observed in rostral NCM, activity was probably influenced by direct inputs from Field L2a (Vates et al., 1996).

Selective NCM neurons must be able to temporally integrate auditory information in order to assess the nature of the stimulus and respond appropriately to zebra finch, and more specifically, forward zebra finch song. It is not known if this temporal integration occurs over the duration of the stimulus, suggesting a time course of several hundred milliseconds, or on a smaller time scale of tens of milliseconds for small groups of syllables. Ribeiro et al., (1998) showed in the NCM of canaries that habituation of expression of the molecular marker ZENK to playback of two

whistles, played singly, did not continue when the two notes were played in combination. Interestingly, the expression pattern for the combination playback was different from the additive patterns of the two whistles individually. These results suggest NCM does not process complex stimuli on a syllable to syllable basis. Certainly, habituating activity would suggest integration times on the order of the length of the stimulus (1.5-2 seconds) since activity decreases continuously over the course of a presentation. Lewicki and Arthur (1996) demonstrated the presence of temporally combination sensitive neurons in Field L, including L2a, to syllable pairs, though global responses to whole songs (both forward and reverse) were indistinguishable.

Molecular and electrophysiology studies focusing on habituation did not demonstrate any differences in responses between males and females (Mello, et al, 1992, 1994, 1995; Chew, et al, 1995, 1996a, 1996b). However, NCM recordings at habituating sites in adult males show habituating activity only when the bird is listening to playback of his vocal output compared to when he is singing (see Chapter 3). NCM may have an additional role in auditory processing and feedback in singing males. If NCM's

function is as a large capacity memory system, as proposed by Chew et al., (1996a), it is conceivably the storage site of a BOS template. When auditory feedback is continuously perturbed for a period of several weeks, BOS is altered (Woolley and Rubel, 1997; Leonardo and Konishi, 1999). However, when feedback is returned to its normal state, the original BOS returns suggesting that a template exists somewhere in the brain. Lesion studies could be performed in conjunction with such experiments to determine if the recovery of normal song requires NCM.

While no NCM sites were found to be exclusively selective to BOS, preferential activity for zebra finch stimuli was found in more caudal regions. Species-specific ZENK activation has been reported previously (Mello et al., 1992), though expression was less restricted than we observed here using electrophysiology. Stimulus discrimination may be occurring in a step-wise manner with the filtering of non-species sounds emerging in stages in NCM. The transformed auditory signal could then be relayed to the song system or other areas requiring conspecific auditory information. Whether BOS selectivity in HVC is relayed via NCM or other sites afferent to HVC (i.e. Nif, or HVC shelf) could be determined by

recording from HVC in animals with lesions in NCM, HVC shelf, or Nif. As well, one could record from auditory regions just upstream of HVC, such as HVC shelf and Nif to determine if BOS selectivity occurs in these nuclei. Shelf recordings were done in anesthetized very young white-crowned sparrows (Whaling et al., 1997) and revealed no distinction in activity between con- and heterospecific song, in contrast to HVC. However, such responses may change in awake behaving animals, and/or as the bird matures.

The region(s) of the songbird brain responsible for selecting ethologically important auditory stimuli from the environment are only beginning to be identified and understood. In addition to song learning and maintenance, proper recognition of conspecific song and BOS is important for mating and male-male interactions. We have now added an additional step in the bottom-up processing of complex auditory stimuli. While studies focusing on Field L suggested that the auditory processing of complex stimuli did not occur in the auditory pathway, these data suggest that NCM may be a parallel station where auditory information is further assessed for behavioral relevance. NCM appears to be a nucleus involved in the transition from general to specific auditory processing. This raises

the possibility that the BOS-restricted auditory responses observed in the song nuclei are the result of filtered inputs from the ascending auditory pathway.

CHAPTER 3: Singing-Specific Responses in NCM

How do birds maintain the ability to produce the same stereotyped song throughout their lifetime? Earlier, it had been thought that once song crystallized, an auditory component was no longer necessary (Konishi, 1965; Bottjer and Arnold, 1984). More recent work has shown that auditory feedback is required since the deafening of adult male zebra finches induces song degradation, albeit over a much longer time period than observed in juvenile birds (Nordeen and Nordeen, 1992; Lombardino and Nottebohm, 2000). Areas involved in song production exhibit BOS-selective auditory activity, but many of these responses are found in anesthetized animals, and fail to appear in the awake, behaving bird (Schmidt and Konishi, 1998; Margoliash, 1998; Hessler and Doupe, 1999). In fact, auditory responses in these areas are inhibited during singing (McCasland and Konishi, 1981; Hessler and Doupe, 1999b) which throws into doubt the role of the song nuclei in auditory feedback.

From molecular studies, NCM is known to show sustained activity of a marker expressed in association with neuronal

depolarization in continuously singing birds, but not in birds that have been deafened (Jarvis and Nottebohm, 1997). Thus far, I have demonstrated the presence of auditory selectivity for species-specific song in NCM of the ascending auditory pathway. To investigate the involvement of NCM in auditory feedback, multiunit neuronal responses were compared during singing versus listening to vocal output. Responses differed depending on whether the sites were nonhabituating, or habituating during passive song listening. Nonhabituating sites showed no obvious distinctions between singing and playback. In contrast, during playback, habituating sites did not habituate during singing and instead assumed a firing pattern consistent with specific points in the song. This is the first demonstration of singing-specific neuronal activity in the auditory pathway.

Results

Multiunit neuronal responses were recorded at six sites in four actively singing adult male zebra finches fitted with chronic electrodes. Birds sang song directed towards a female, or

undirected song for 50-70 bouts. At the same time, the bird's vocal output was stored on tape. Within 30 minutes after ceasing recording of the neural and vocal signals, the bird's vocal output was played back to him and multiunit activity was again measured, this time during passive listening.

As described in Chapter 2, responses in NCM to recorded stimuli can be placed into two categories: nonhabituating and habituating. At nonhabituating sites, responses during active singing did not differ from responses to taped playback, whereas sites that habituated during passive listening no longer habituated when the bird sang.

Nonhabituating sites are characterized by responses dependent upon acoustic (i.e. harmonic content, frequency and amplitude modulation) and temporal features for activation. These sites may show some decrease in activity over the course of playback, but responses remain consistent to specific stimulus notes. Habituating sites initially respond robustly, then fade over the course of several presentations. Within a presentation, activity is most robust to the initial portion of the stimulus and then declines.

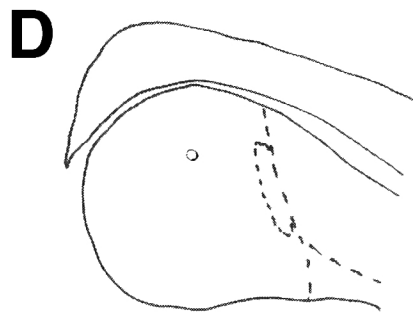
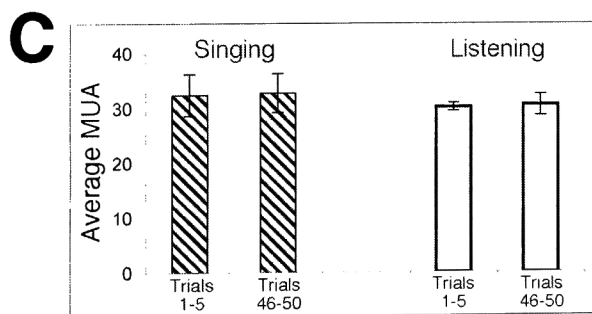
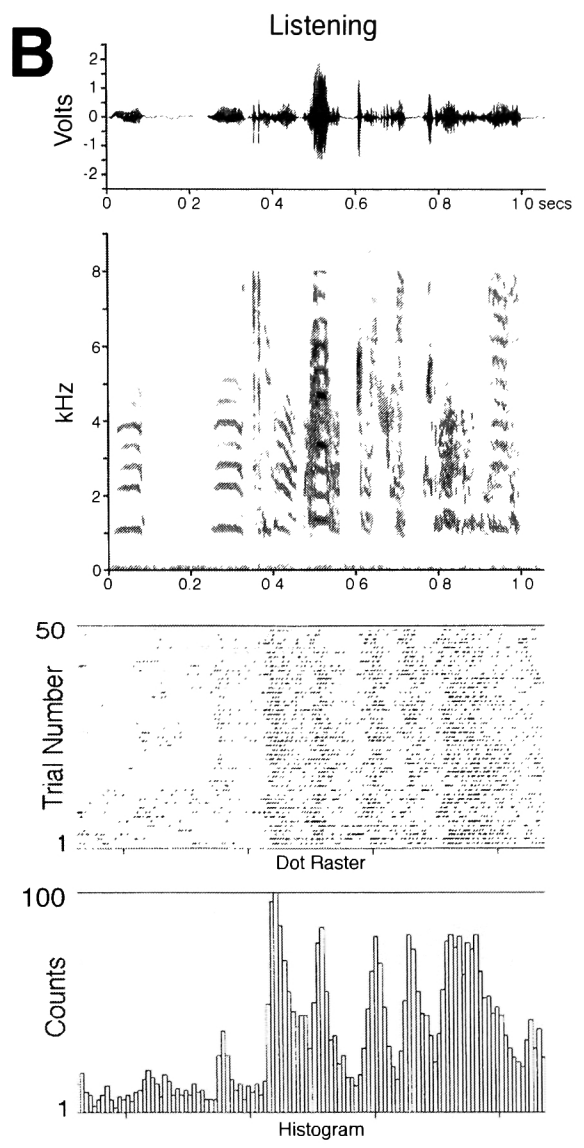
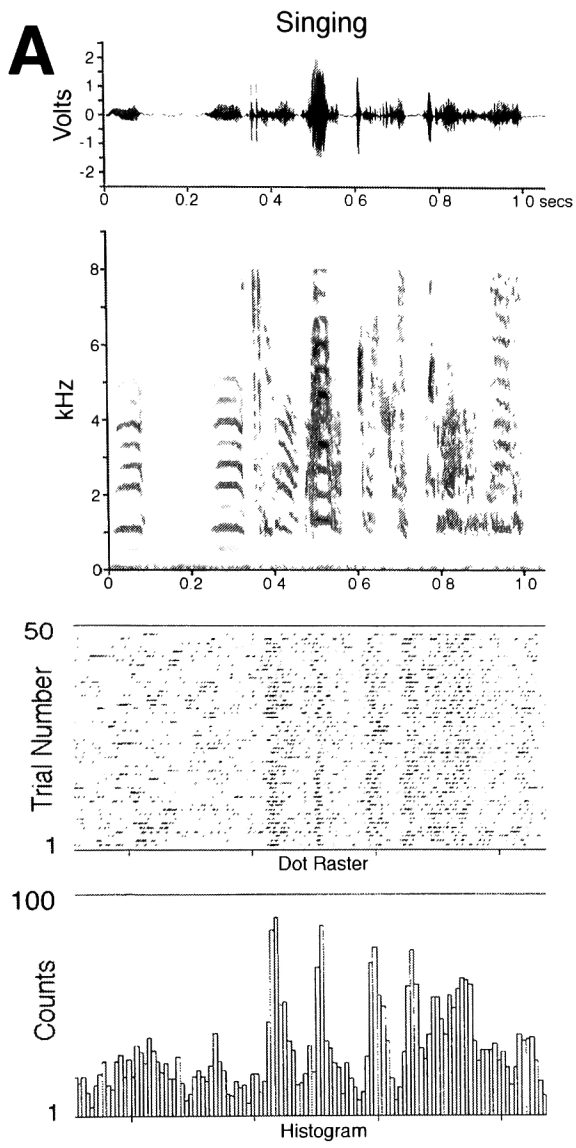
Responses in Nonhabituating Sites

Neuronal responses during singing versus playback in nonhabituating sites (4 sites in 3 birds) showed remarkably similar patterns of activity. The example illustrated in figure 10 was recorded from a bird singing undirected song. For the two response panels, the amplitude diagram in the uppermost trace represents stimulus volume. Immediately below, the sonogram separates the stimulus into its component frequencies, with respect to time. Multiunit activity is shown in two forms: dot raster and histogram analysis.

The dot raster and histogram patterns for singing (figure 10A) and listening (figure 10B) are striking in their likeness. Response peaks occurred at almost identical points in the song. The site illustrated was originally identified during playback of digitized stimuli as vigorously responding to all song types and most song characteristics. The latter neuronal behavior was also maintained during singing. Both singing and listening produced robust activity patterns to harmonically complex, rapidly

Figure 10. Singing versus listening responses in a nonhabituating site.

Neuronal recordings were made during active singing (A) and during playback of vocal output (B). Diagrams from top to bottom show the amplitude envelope of the song, song sonogram, dot raster and histogram analysis of activity. This recording was done in a site that had responded robustly to all stimuli during passive listening experiments. Both singing and listening produced very similar robust activity patterns to harmonically complex, rapidly modulating notes. Unmodulated harmonic stacks of stable amplitude elicited much weaker activity. The slight variations in response pattern are most likely due new units(s) coming into the range of the electrode. C) The average MUA was measured for Trials 1-5 and Trials 46-50 over a 400ms period indicated by the gray bar above each sonogram. The change in activity from start to end of each session was not significant for either singing or listening ($p>0.05$). D) The recording site was located in the dorsal half of NCM towards the rostral extent. Dorsal is up and rostral to the right.



modulating notes. Unmodulated harmonic stacks of stable amplitude elicited much weaker activity. Slight variations in response pattern were sometimes observed. These differences were most likely due electrode movement causing new unit(s) to come into range at the recording site, though behavior-specific influences cannot be ruled out. Similar patterns of activity were observed regardless of whether birds sang directed or undirected song.

The change in activity levels over the course of each experiment is pictured graphically in figure 10C. The average MUA for Trials 1-5 and Trials 46-50 were calculated for the 400ms segment of song highlighted by the gray bar above each sonogram. The MUA remained essentially the same for both singing (32.4 events \pm 3.8 SEM for Trials 1-5, 32.8 events \pm 3.5 for Trials 46-50; hatched bars figure 10C) and listening (30.2 events \pm 0.7 for Trial 1-5, 30.6 events \pm 1.9 for Trials 46-50; open bars figure 10C). The early and late trial values were not significantly different for either condition ($p > 0.05$, unpaired student's t-test). The average number of MUA for all 50 bouts during the 400 ms interval was not significantly different for either behavior (30.7 events \pm 0.9 and 31.02 events \pm 1.0, for singing and listening, respectively; $p > 0.05$).

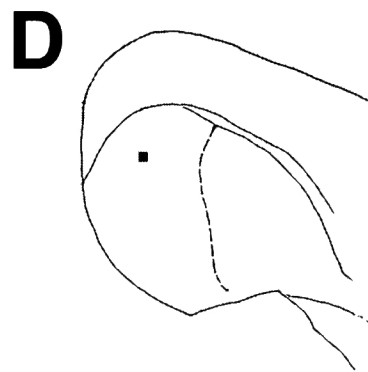
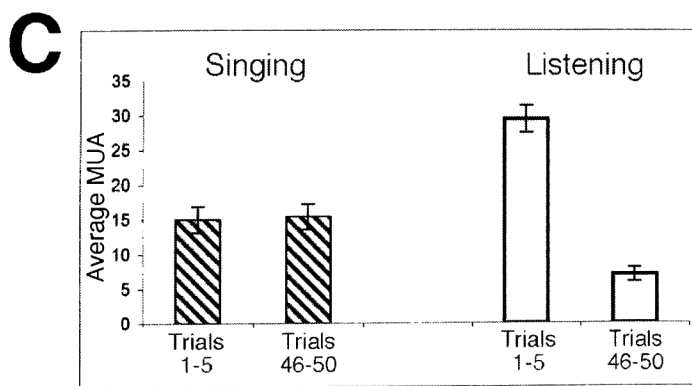
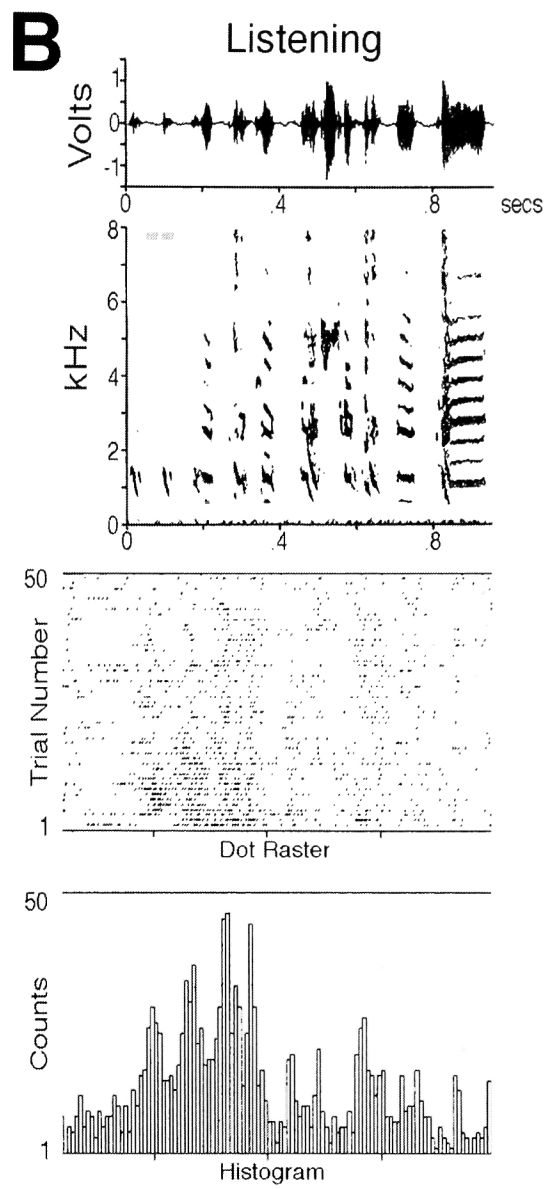
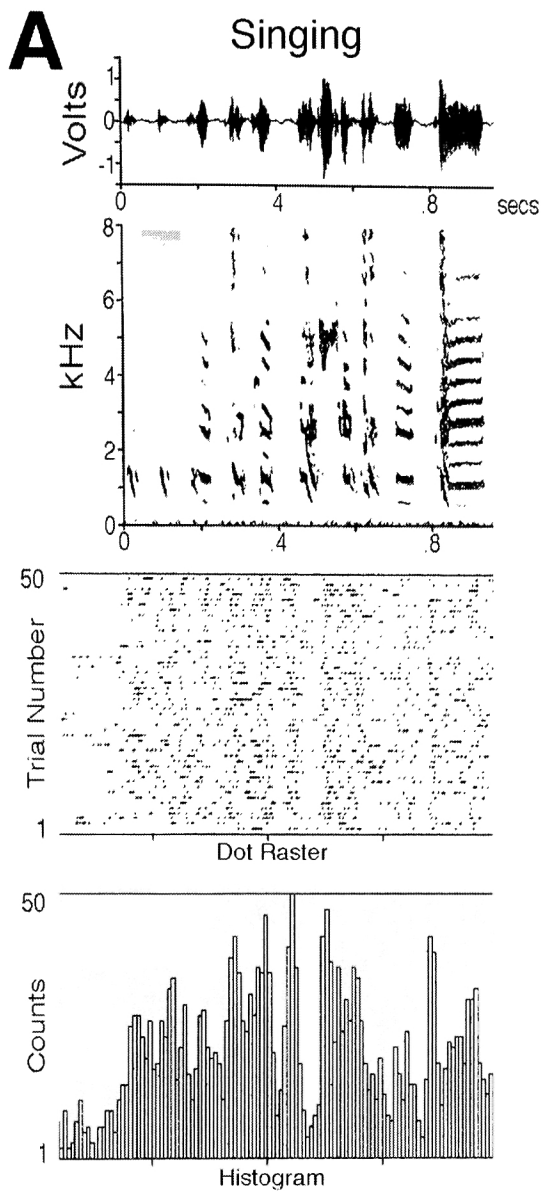
Thus, auditory activity does occur in response to singing at nonhabituating NCM sites providing evidence for the involvement of these cells in the auditory feedback of vocalizations. These responses are highly similar to the responses observed for playback of recorded singing. This suggests that nonhabituating sites are using the same acoustic and temporal cues in the processing of auditory feedback as is observed for played BOS. Such responses are likely to be purely auditory with no singing-related component.

Responses in Habituating Sites

A behavior-dependent difference in NCM was discovered when sites that habituated to song playback did not show a similar decline in activity during singing (2 sites in 1 bird). The site featured in figure 11 is from the same bird as in figure 8. Responses during singing (figure 11A), though somewhat noisy, remained sustained from trial to trial, and appeared to occur throughout the song at specific points, not just to the early notes. In contrast, responses during passive listening (figure 11B) showed no such stereotyped response, and instead exhibited vigorous

Figure 11. Singing versus listening responses in an habituating site.

Diagram organization is as described in Figure 10. This recording was done in the same bird as depicted in figure 8. (A) Activity while the bird was singing did not habituate and instead was sustained throughout the song and from bout to bout. (B) Habituation occurred during passive listening which was characterized by vigorous auditory responses to the early presentations and the initial notes of the stimulus followed by decreased activity. (C) The average MUA was measured for Trials 1-5 and Trials 46-50 over a 300ms period indicated by the gray bar above each sonogram. Activity did not change significantly during singing ($p>0.05$), unlike during listening where response dropped by 76% from the start to finish of playback (significance $p<0.0001$). (D) The recording site was located in the dorsal area around the center of NCM. Dorsal is up and rostral to the right.



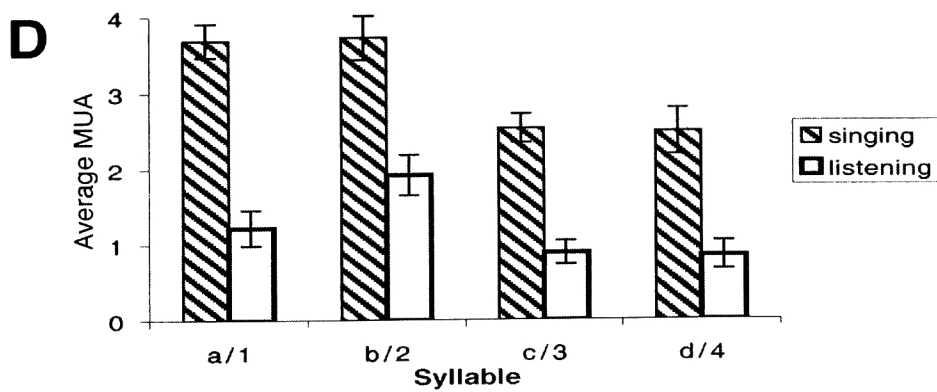
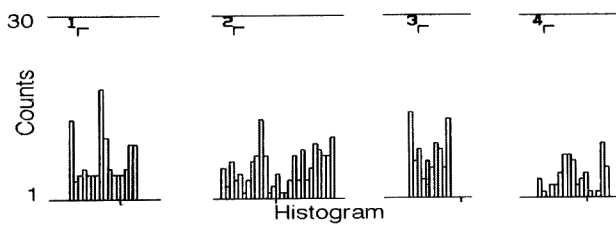
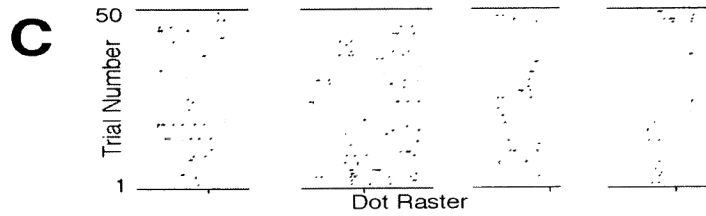
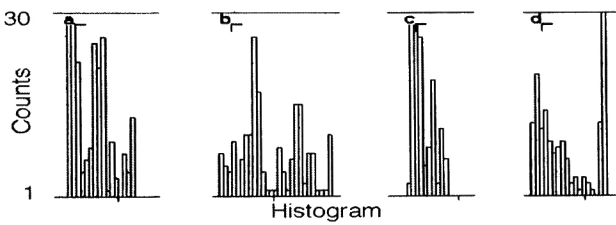
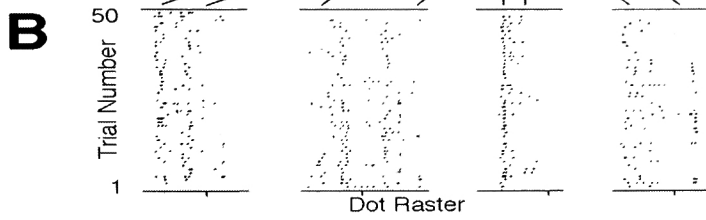
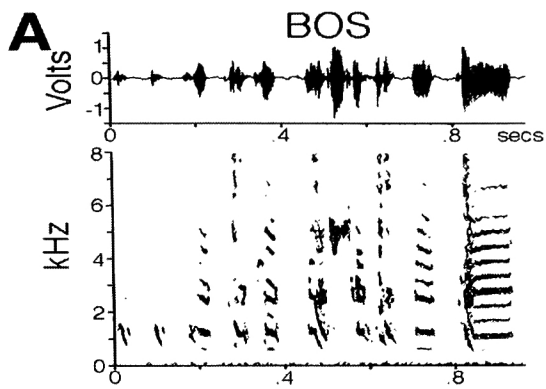
activity only during the initial presentations and initial notes of BOS playback, followed by decreasing activity. No premotor activity was observed prior to singing suggesting these responses were not able to anticipate and instead could only respond to vocal production.

The discrepancy between singing and listening is further emphasized by comparing the change in the average MUA for Trials 1-5 and Trials 46-50 during the 300ms period of song highlighted by the gray bar above each sonogram. The average MUA during singing remained essentially the same (15.0 events \pm 1.9 for Trials 1-5; 15.4 events \pm 1.8 for Trials 46-50; $p > 0.05$). During listening, activity dropped by 76% from 29.4 events \pm 1.9 for the first five trials, to 7.0 events \pm 1.0 for the last five trials (significance $p < 0.0001$). Interestingly, the mean overall MUA was similar for singing (14.2 events \pm 0.7) and listening (12.5 events \pm 1.2; $p > 0.05$), although one would not expect this to be the case were singing and playback to continue since activity for the latter should remain habituated for additional presentations.

Due to the difficulty of recording in singing birds, most of the data for singing and listening were collected as separate events.

Figure 12. Detail of syllable responses in a habituating site.

Neuronal recordings were made concurrently during active singing (B) and playback of vocal output (C) in a site that has already habituated to playback of BOS (A). Interleaved data were separated into the two behaviors. Responses for syllables in the motif were individually aligned to highlight any activity patterns that might not have been as apparent due to intersyllable time variability. This recording was done in the same bird depicted in figures 8 and 11. Diagrams from top to bottom show the amplitude envelope of the song, song sonogram, the dot raster and histogram analysis of activity during singing and listening, and a graph of the average number of counts per trials for each behavior. B) Activity during singing occurred throughout the course of the experiment at specific points that coincided with the start or end of some syllables. C) Activity during passive listening had already habituated resulting in disordered and weak activity overall. D) For each corresponding syllable, activity was always significantly higher during singing than listening ($p < 0.0001$).



However, in a subsequent experiment, I was fortunate to be able to record responses for both behavioral modes concurrently (figure 12). In order to better illustrate the differences in activity, interleaved signals were separated into those that occurred during singing, and those that occurred during listening. Habituation was observed to have already been established to song playback (figure 12C), while activity during singing was sustained (figure 12B). To overcome the intersyllable time variability that occurs during natural song, individual syllables were aligned, instead of aligning the beginning of each song bout. A pattern of response emerged and robust activity was observed during singing compared to listening which exhibited responses that were much weaker and more disordered. The average number of counts per trial was quantified for each syllable (figure 12D). In each case, response during singing was significantly higher than during listening ($p < 0.0001$). Activity appeared to coincide with the start, or end of a syllable and occurred at specific points in the song. The response did not seem to be driven by specific spectral characteristics, or frequency and amplitude modulations, unlike the activity observed at nonhabituating sites.

Discussion

For the first time, I have shown a singing-specific response in an auditory region of the zebra finch brain. The differences between nonhabituating and habituating sites in auditory processing were highlighted in these studies by comparing responses during singing and listening to vocal output. While nonhabituating sites showed little change between these two behavioral states, sites that habituated to song playback did not habituate during singing. Instead, responses were characterized by a regular pattern coincident with specific points in the song.

Are the responses observed during singing purely auditory? Certainly in the case of nonhabituating sites, response can be regarded as such owing to the concordance in pattern of activity between singing and listening. Auditory feedback is necessary for song maintenance (Nordeen and Nordeen, 1992) and auditory responses are not observed in song nuclei during singing (McCasland and Konishi, 1981; Hessler and Doupe, 1999b). However, the nature of the activity in nonhabituating sites suggests that auditory information from singing and passive listening is regarded in the same manner. In other words, nonhabituating sites

may not be able to distinguish between incoming auditory information that is self-generated or from an external source.

While these sites are clearly involved in relaying auditory feedback, they do not appear to be specific since few distinctions in response are made between external and self-generated vocalizations.

For sites previously found to habituate to played stimuli, it is less clear from where the new singing-related activity arises, and what its function might be. Clearly, responses are influenced by the act of singing. However, the response pattern could be either a reflection of activity as exhibited by the song nuclei, or a disinhibition of auditory response during singing. Pre-vocalizing activation is observed in most of the song regions (McCasland, 1987; Hessler and Doupe, 1999b) and yet is not seen in these sites, which suggests that the response in habituating NCM is not the same as in the song nuclei. To test this hypothesis, auditory input could be removed by deafening birds. The persistence of activity would point to a motor element in the response while a loss of response would be attributed to a loss of auditory feedback. Habituation does not appear to be the result of a uniform depression of the site. Rather, lack of responsiveness seems to

depend on the nature of the stimulus. Thus far, the underlying mechanisms resulting in this activity have been found to be influenced by context – in one case, stimulus novelty, in the case highlighted in this thesis, internal versus external stimulus source.

An auditory function for cells showing differential activity might be to “pay attention” in order to monitor that song is being produced correctly. Such an error-checking mechanism might analyze for acoustic, and/or temporal accuracy. The fact that response occurs at specific points in the song and does not appear to be coincident with acoustic features, suggests that these neurons may be more concerned with temporal precision. Both possibilities could be tested by temporary manipulation of auditory feedback. Studies were begun using helium inhalation to alter vocal output (appendix 1). If singing-related activity at habituating sights is responding to acoustic features specific to BOS, a helium-influenced change in spectral frequency might effect a change in response pattern (Nowicki, 1987). Temporal-specific responses would be more difficult to address. A temporally altered song (e.g. scrambled notes) could be played via a speaker or headphones to singing birds. However, this approach is complicated by

competition from the animal's vocalizations. Any new responses would have to be extracted from the original activity.

A template copy of BOS is believed to exist based on studies that altered auditory feedback resulting in abnormal BOS. The return of normal hearing allowed the original BOS to resurface (Woolley and Rubel, 1997; Leonardo and Konishi, 1999). If NCM is involved in checking that song is produced correctly, it could be comparing vocal output with a stored template. This raises the question of whether the region might actually store the template copy. As discussed in Chapter 2, habituating sites in NCM exhibit a large capacity memory storage for familiar songs (Chew et al., 1996a), and might be capable of storing a copy of the BOS template.

Singing-related activity should also be accompanied by anatomical inputs from the song circuit to NCM. One possible connection lies in paraHVC - a structure extending from the medial portion of HVC. ParaHVC receives afferent connections from HVC and mMAN, and is thought to project to the dorsal portion of caudal NCM (Foster and Bottjer, 1998). Habituating sites are concentrated in the caudal extent of NCM (Chew et al., 1995;

Chapter 2) potentially placing at least some of them in position to receive information from HVC via pHVC. However, retrograde anatomical connections from dorsocaudal NCM to pHVC have yet to be shown which leaves the validity of this connection yet to be proven. As well, the sites from which recording took place appear to fall outside the proposed region of pHVC termination in NCM. Another potential source of song pathway inputs to the auditory system is from the HVC shelf to the RA cup, which surrounds RA (Mello et al., 1998b). The RA cup projects to MLd and the Ov shell which sends connections directly into Ov. No connections from HVC to shelf, or RA to cup have been detected, but the close apposition of these regions might allow for singing information to reach the auditory system. The signal that the bird is singing might be relayed by the shelf or cup and carried to Ov. Such a signal would then move up the ascending auditory pathway where it would influence responses in NCM. Recordings in HVC shelf and RA cup during singing should be done to determine if responses in these areas are auditory and/or song-related.

By presenting the same vocalizations during playback as produced by the bird while singing, the variability due to song

“jitter” was taken into account. Jitter is defined as the small changes in frequency modulation and timing that are observed during normal singing between a bird’s successive renderings of BOS. Jitter does not seem to affect the auditory perception of BOS during playback in NCM based on the responses observed.

Habituating sites still habituated to taped song (figure 11A) suggesting small changes from one rendition to the next did not hamper recognition of a familiar motif. As well, the auditory responses to digitized and taped BOS were very similar (data not shown).

Differences in molecular and electrophysiological activity are known to occur in Area X and IMAN when birds direct singing towards a female, compared to singing in an undirected manner (Jarvis et al., 1998; Hessler and Doupe, 1999a). Directed song increases in speed and motif number and functions as a courtship behavior, while undirected song is thought to be for establishing contact with birds who might be in the region, and for rehearsal of song. It is not known if there is a change in auditory perception with this change in social context. Perhaps birds listen more carefully to vocal output during undirected song to ensure that a

good copy will be produced when singing to a female.

Dissimilarities in nonhabituating sites were not observed when the birds sang directed or undirected song suggesting that responses in these cells are independent of behavioral state, and solely reflect auditory information. Future studies should compare activity during directed and undirected singing in sites that exhibit differences between listening and singing to determine if they might also show a distinction for vocalizations involved in direct social interaction.

A novel singing-specific activity has been recorded in NCM sites that otherwise habituate to auditory stimuli during passive listening. Auditory feedback is necessary for proper song maintenance, although auditory activity is not present during singing in the song nuclei and singing-specific responses had not been previously demonstrated in the auditory pathway. While these responses in NCM have yet to be determined to be auditory, motor, or some other behavioral modality, they provide the possibility of NCM being a long sought-after region of the brain that assesses the accuracy of produced song.

CHAPTER 4: CONCLUSIONS

The auditory system is constantly receiving sounds that must be interpreted for importance to the animal. In addition to the information coming from the surroundings, there are also self-generated vocalizations to be assessed. This is particularly true in the zebra finch which communicates via learned song. The work presented in this thesis has investigated the function of the zebra finch caudomedial neostriatum in its ability to resolve behaviorally relevant auditory information. I have characterized the physiological responses of NCM both during listening and singing. The complexity of this nucleus has provided challenges in establishing its role in auditory perception. Indeed, the variety of responses has revealed NCM to have not just one function, but many.

During playback of BOS, rev BOS, conspecific song and an excerpt of canary song, NCM was found to range in response from robust for all stimuli, to selective for zebra finch song. Sites that exhibited robust activity to all stimuli tended to be found towards the rostral region of NCM, while selective sites were more caudal.

Many of these sites responded best to harmonically complex syllables composed of rapid frequency modulations. These acoustic features are characteristic of portions of zebra finch song, and may be the cues used by the auditory system to recognize conspecific sounds. A subset of sites, termed habituating, also demonstrated varying degrees of stimulus selectivity were found in the same caudal region as nonhabituating selective sites. These neurons were not activated by specific acoustic elements. Instead, these cells responded preferentially to the first few presentations of a stimulus, and to the early portions of each presentation.

Habituation was not found to occur during singing in any of the sites recorded. Activity was observed as either almost indistinguishable from nonhabituating passive listening responses, or specific for song production. Singing-specific activity has never before been observed in an auditory region of the songbird brain. Unlike nonhabituating sites, which responded best to acoustical cues, singing-related responses coincided in a stereotyped manner with points in the song that did not appear to share specific harmonic characteristics.

All of these responses, though wide-ranging, point to a role for NCM in the processing of complex, behaviorally relevant auditory information during listening, and singing. The data also leave many unexplored avenues for further work.

Future studies can expand upon the nature of the specificity of response in NCM and the auditory regions that follow. If auditory processing follows a step-wise organization with increasing selectivity at successive stations, progressively more filtering should be observed at cmHV, clHV, Nif, and HVC shelf, compared to NCM. This would be best studied by recording responses simultaneously in two or more nuclei to a set of played stimuli. Chronic recordings are limited by the small number of electrodes that can be used at any one time, and by displacement of the electrode from the recording site due to movement which happens often and makes holding units difficult. Instead, the use of multielectrodes in the restrained animal would probably be more effective. Different kinds of stimuli can also be tested, for example pure tones, manipulated song, and songs from other songbird species to define better the degree of selectivity at the various sites. Recordings could be performed in anesthetized animals to isolate

single units, though anesthesia is known to alter auditory responses in some of the song nuclei (Schmidt and Konishi, 1998; Dave et al., 1998) and might also influence activity in the ascending auditory pathway.

More investigations must also be made into the singing-specific response that emerged in NCM. The presence of auditory activity that occurs only during singing has yet to be proven, and must, in order to provide the first step of a functional mechanism for auditory feedback. Unlike the stimulus playback studies, singing-related experiments can only be done using a chronic electrode approach since the animals must be comfortable enough to vocalize. Simultaneous recordings in multiple locations along the auditory pathway can be carried out to assess the change in the neural signal (if any). Attempts to follow the progression of singing-specific auditory responses might lead to information on where the activity reaches the song system. Electrodes could also be coated in retrograde and anterograde tracers to try to obtain afferent and efferent connectivity data specific for the site.

Do these responses change during development? Initially unselective, HVC and the anterior forebrain pathway nuclei of

anesthetized birds exhibit emerging selectivity for BOS and tutor song as they enter the song learning stages and increasing BOS-preference that coincides with the time of song crystallization (Volman, 1993; Solis and Doupe, 1997). A similar phenomenon might also occur in NCM if the selective responses observed are the result of tuning by external stimuli during juvenile development. While there is some evidence that recognition of conspecific sounds in songbirds is hardwired (Marler and Tamura, 1964), molecular and physiological studies in adult birds have shown activity in NCM to be influenced by experience (Mello et al., 1995; Chew et al., 1995, 1996a, 1996b; Stripling et al., 1997). It would be a challenge to devise methods to track response changes in a particular site as the bird, and its brain are growing. As well, it might be difficult to distinguish any changes in response behavior as being due to developmental effects, or the general heterogeneity of NCM. If the recognition of species-specific sounds is learned, then manipulation of the aural environment of young birds, by exposing them only to heterospecific song for example, should produce sites that are selective for the anomalous stimuli. On the other hand, if such activity is innate, then zebra finch specific responses will remain.

Another role as yet unexplored role for NCM may be in sound localization. The formation of an auditory space map is known to be necessary for locating sounds (reviewed by King, 1999). Songbirds (like the zebra finch) that live in social colonies can determine when the song of a neighbor is being played from a different venue than usual (Brooks and Falls, 1975; Godard, 1991). All of the studies described in this thesis were done from a fixed sound source. It would be interesting to test if stimuli played from speakers placed in different locations around the bird induce changes in activity, especially in sites that habituate to playbacks since those cells appear to be able to recognize familiar sounds. Both electrophysiological and ZENK expression experiments could be employed to investigate this hypothesis.

Finally, a relationship between physiological activity and ZENK expression has yet to be firmly established. Based on the pattern of ZENK induction, molecular activity appears to follow the behavior of physiologically habituating sites. Specifically, expression demonstrates some degree of selectivity to species-specific stimuli (Mello et al., 1992), induction is strongest to novel songs (Mello et al., 1995), and ZENK levels remain sustained, in an

auditory dependant manner, during singing over a period of 50-70 song bouts (Jarvis and Nottebohm, 1997). A link between the two types of cellular behavior might be best achieved by recording from single units using glass electrodes. The electrodes can be filled with a dye, such as diI, that marks the cell being analyzed. Immediately after recording, the brains can be prepared for either *in situ* hybridization or immunocytochemistry assessment of ZENK activation and the degree of gene expression at the site compared to the physiological behavior.

Together, these proposals, and the work leading up to them, highlight how much there is still to learn about NCM and the ascending auditory pathway as it relates to the perception and processing of auditory information. Hopefully, the studies described in this thesis will provide the basis for future, fruitful work.

APPENDIX: The Effect of Helium Inhalation on Zebra Finch

Song

The sites in NCM that exhibited singing-specific activity called for further investigation. It is not yet known if these responses are strictly auditory, motor, or some thereof. To address the issue of whether auditory input might be contributing to the observed activity, I have devised a system to manipulate vocal output by delivering a gas mixture of 80% helium/20% oxygen (heliox) to actively singing birds. Air where the nitrogen content has been replaced by helium has been previously reported to alter vocalizations in oscine songbirds by increasing the amplitude of higher frequencies (Nowicki, 1987). Unlike previous studies that altered auditory feedback (Nordeen and Nordeen, 1992; Wooley and Rubel, 1997; Leonardo and Konishi, 1999), helium inhalation would induce temporary effects of controllable duration. Longer lasting manipulations to auditory feedback presumably cause changes in song system neuronal activity as vocal output becomes altered. Such changes might also influence responses in the auditory pathway. By introducing only short-term changes, the

motor system may not have the opportunity to adapt to the modified vocal output and any differences in the neural signal could be solely attributed to changes in auditory feedback. Also, since auditory perception is altered at the level of vocal output, instead of being played over a speaker, there is no competition from the bird's singing.

Neuronal and auditory recordings were made in the chronically implanted NCM of an actively singing bird housed in the chamber used for all chronic recordings. A custom designed plastic tent was fitted over the recording chamber. The tent contained an opening for a tube (75 mm inner diameter; Nalgene, Rochester, NY), leading from a tank that held a compressed gas mixture of 80% helium and 20% oxygen (Matheson Gas, Rutherford, NJ). After 30-50 song bouts had been recorded in normal atmosphere, the heliox mixture was released continually inside the chamber at a fixed pressure of 11-12 kPa.

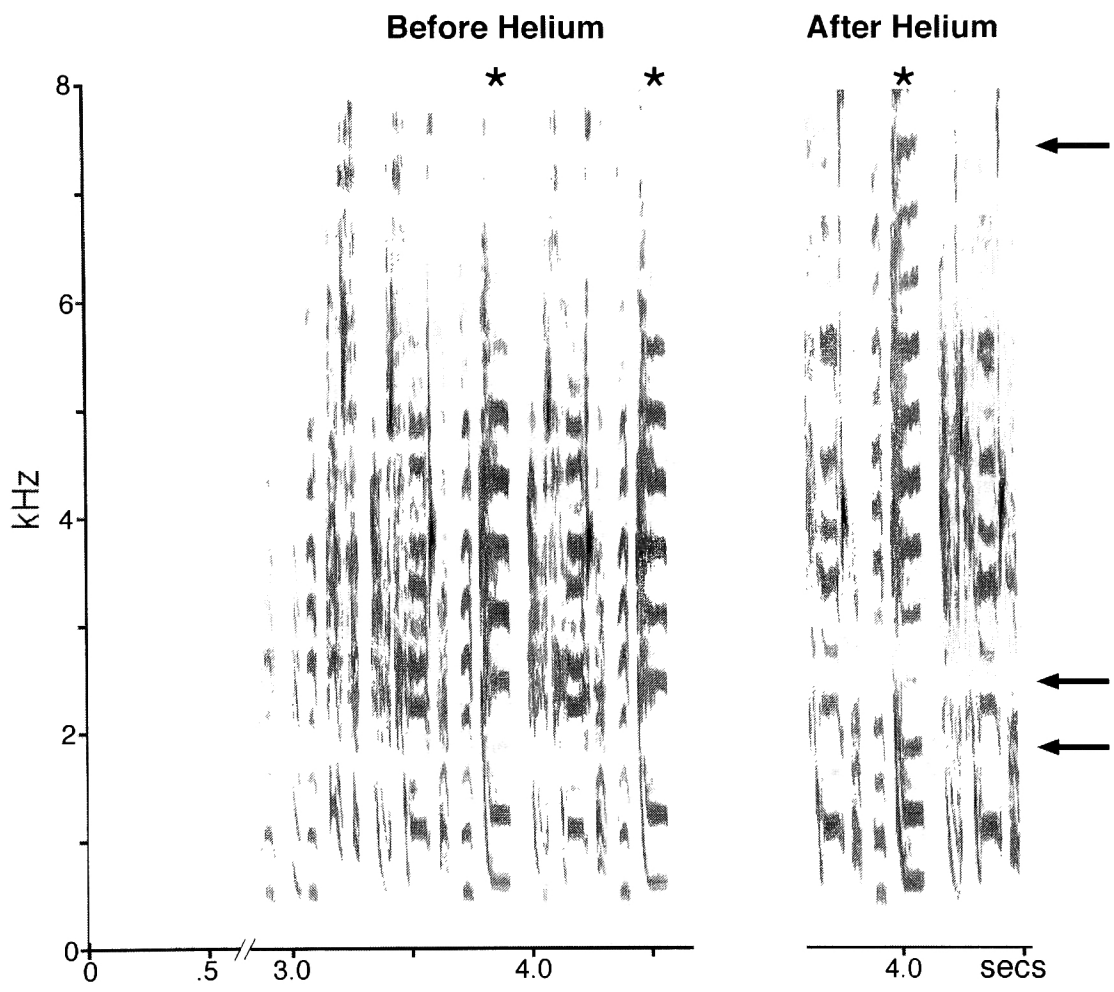
Unfortunately, birds were not as inclined to sing in heliox, and only produced a few bouts over a period of 1-2 hours. This precluded any opportunities to record from NCM. The reluctance to sing was probably due to the fact that helium conducts heat

approximately six times faster than compressed air (Rutkowski, 2000) and as a result, the birds may have become uncomfortably cold. Heating the chamber, and/or the heliox during delivery may ameliorate this problem. As well, a more airtight chamber, and more expeditious method to introduce the gas would speed the process of vocal manipulation. One possibility would be to have an air bladder filled with heliox attached to the outside of the chamber. When it was time to change the breathing environment, a valve from the chamber to the outside atmosphere would be opened and the contents of the bladder released into the tent thereby displacing the air inside. The new environment could then be maintained by a steady flow of gas (suggested by D. Vicario, personal communication).

Changes in the quality of song were detected within 15 minutes after the introduction of heliox. This was most noticeable in the notes composed of stable harmonic stacks (figure 1, notes marked by asterisks). Confirming the study of Nowicki (1987), the addition of harmonic overtones and a shift in amplitude to emphasize higher frequencies became evident, though the fundamental frequencies did not appear to be altered (arrows for

Figure 13. Sonograms of zebra finch song before and after helium inhalation.

Song was recorded in normal air, then in an atmosphere of 80% helium/20% oxygen. The effect of heliox on song is best observed in the syllables composed of stable harmonic stacks highlighted by the asterisks. In heliox, higher frequencies became more prominent (arrows) owing to an upward shift in amplitude. No change in the fundamental frequency was apparent.



notes marked by asterisks). As well, song became noticeably quieter in the new atmosphere (data not shown). Birds could be maintained in this environment for periods of several hours without any apparent ill effects.

Though I could not obtain any clean electrophysiological recordings while the bird was breathing helium, I was able to determine the efficacy of the gas mixture on song. Whether the changes in vocal output are actually perceived by the bird, and whether NCM is the region that processes the change in perception still remains to be established.

MATERIALS AND METHODS

Animals

Adult male zebra finches (>90 days post-hatch) were obtained from the Rockefeller University Field Research Center breeding colony. All males were implanted with a testosterone silastic (approx 30 μ g testosterone propionate (Sigma)/bird) under the skin alongside the pectoral muscle wall. Animals were housed individually both before and during recording. All protocols approved by the Laboratory for Animal Research Care at The Rockefeller University.

Song Recording

Digitized Stimuli

One week following T-implantation, males were placed individually in acoustic isolation boxes for 48 hours on a 12 hour light/dark cycle. Undirected song was recorded shortly after the lights came on using a Sennheiser ME62/K6-C microphone and

Tascam DA-P1 DAT recorder. Song bouts lasting approximately 1.5-2 seconds were digitized (sampling rate of 20KHz) at approximately the same intensity level using the Signal sound analysis commercial software. Digitized song was also manipulated to produce songs that were completely reversed. A two-second excerpt of canary song (a gift from S. Ribeiro) was also digitized using the Signal program.

Singing versus Listening Stimuli

While recording electrophysiological activity in NCM during singing, vocal output was simultaneously recorded for 50-70 bouts on a Sony DAT recorder (model TCD-D8). Songs were recorded in their entirety.

Surgery

Birds were anesthetized by an intramuscular (M. pectoralis) injection of ketamine (2.5mg/ml)/xylazine (5mg/ml) at a dose of 0.75 μ l/(gm of bird) and their heads placed in a stereotaxic apparatus. The divergence of the central sinus bordering the

forebrain and cerebellum (the zero point) was noted and the future site of recording determined relative to the base of the zero point. A small hole was made on either the left or right side at coordinates: 0.6-0.8mm lateral and 0.6-1.0mm rostral to cover the caudal extent of NCM up to the border of rostral NCM/Field L2a. A single nichrome wire electrode (#7615, A-M Systems, Everett, WA) mounted inside an aluminum microdrive (H. Adams, Caltech Machine Shop; Rockefeller University Instrument Shop) was stereotaxically lowered 0.9mm into the brain. The use of a movable microdrive electrode permitted the recording of multiple sites (at separate times) within each bird. The vertical progress of the electrode allowed monitoring of any changes in site behavior along a dorsal to ventral axis. Electrodes were made of nichrome wire that were rhodium-electroplated at the tip and carried an impedance of 200-800k Ω (measured at 1 kHz). An insulated silver reference ground wire (A-M Systems, Everett, WA) was inserted between the brain and the inner skull layer. The ground and electrode were both attached to an hermaphroditic nanoconnector socket (#NHR06H0-30C3-0.11, Ulti-Mate, Orange, CA) and the entire apparatus was permanently fixed in place on the skull with

Dentsply Caulk Grip Cement (#675570, Milford, DE) with a piece of transparent plastic placed around the microdrive for protection.

Stimuli

Zebra finch song is composed of a series of notes characterized by stacked frequencies that range from 2kHz to 10kHz (figure 3A and C). The frequencies and amplitude may remain stable over the course of the note, or may modulate. Song typically begins with a number of short introductory notes followed by one or more repetitions of the main song motif. Motifs consist of a series of notes fixed in both note type and temporal pattern that are established during song learning and remain largely unchanged during the bird's lifetime. Natural song (as presented during the singing versus listening experiments) displays a certain degree of variability in syllable duration, intersyllable interval, and in the number of introductory notes and motifs sung per bout. Reverse BOS (figure 3B) retains the harmonic features of zebra finch song, but the temporal pattern is now reversed and frequency modulations, normally characterized by downsweeps, now sweep

from low to high frequency. Canary song (figure 3D) is harmonically less complex than zebra finch song. Notes are generally stable in frequency, though amplitude may change over the duration.

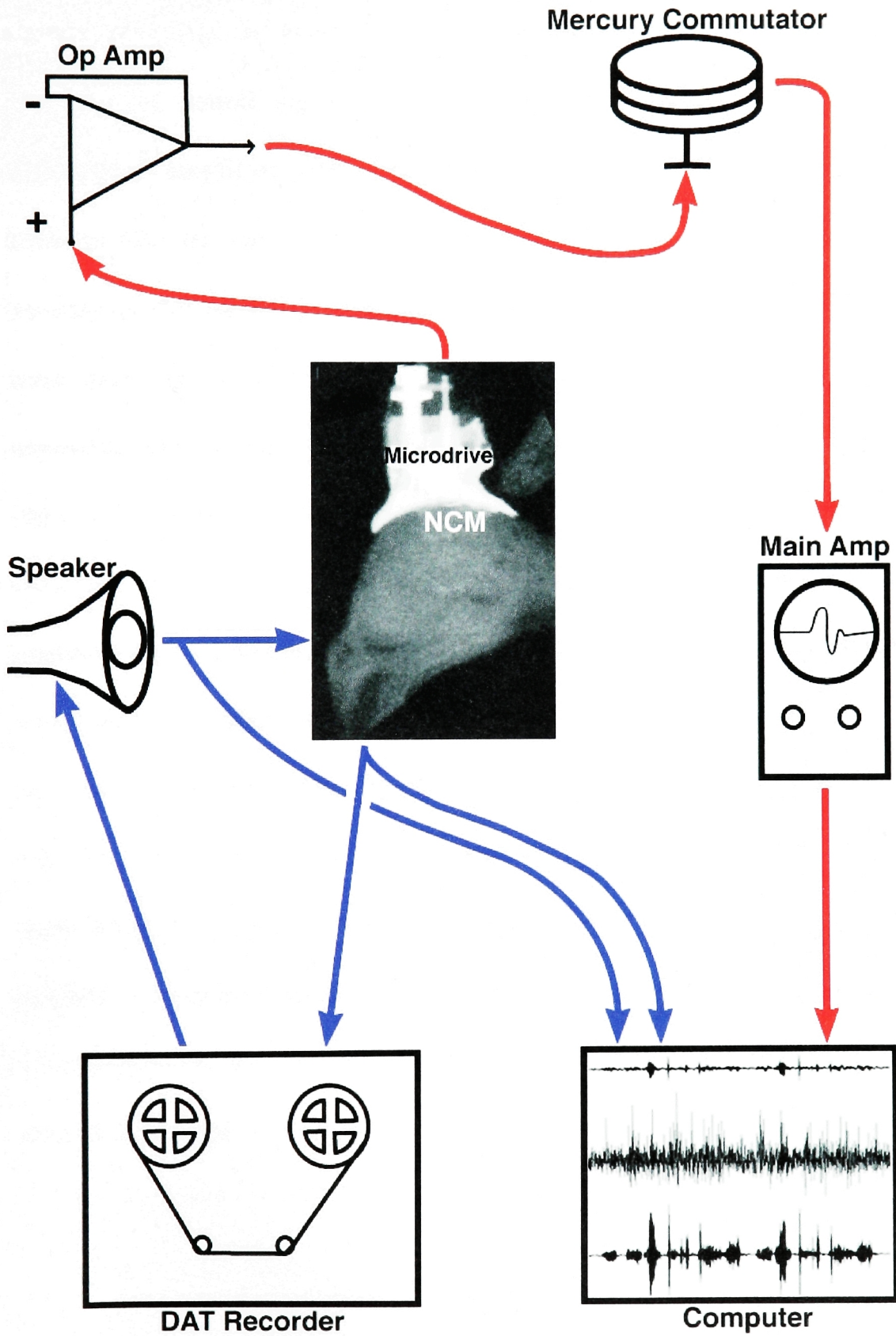
Physiological Recordings

Two days after implantation, birds were placed in an acoustically isolated plexiglass recording cage. Males were primed to sing shortly after being placed in the recording cages by introducing a female within visual range. Priming seemed to help induce the production of undirected song on subsequent days. As illustrated in figure 14, the electrode connector on the bird's head was attached to a small operational amplifier (TL074CPWLE, Texas Instruments, Dallas, TX) which was bridged via flexible PVC coated 38 gauge copper wires (Cooner Wire, Chatsworth, CA) to a nine-channel mercury commutator (H. Adams, Caltech Machine Shop) which permitted movement through a 360° arc of rotation on a horizontal plane. The operational amplifier transformed high impedance neural signals coming from NCM into low impedance

Figure 14. Schematic diagram of the chronic electrode and sound set-up.

Extracellular neural signals (represented by red arrows) were picked-up by a single electrode mounted inside an aluminum microdrive. Electrodes were made of nichrome wire that were rhodium-electroplated at the tip and carried an impedance of 200-800k Ω (measured at 1 kHz). Neuronal information then passed through a small operational amplifier which transformed high impedance neural signals into low impedance signals resulting in greatly reduced movement artifacts. Electrical contact was maintained through a nine-channel mercury commutator which permitted full range of movement. Neural signals passed from the commutator to a differential amplifier (bandpass filtered between 500 Hz and 5 kHz), then were digitized (at 20 kHz) and stored on computer.

Acoustic information (represented by blue arrows) was both presented to the bird and recorded from him. Digitized stimuli (source not shown) were played from a speaker and simultaneously relayed to the with the corresponding neural signals. For singing versus listening experiments, spontaneous vocal output was stored on DAT recorder, and (together with neuronal activity) on computer. The audio recording was then played back to the bird, and the sound and neuronal information sent to the computer.



signals resulting in greatly reduced movement artifacts.

Extracellular neural signals passed from the commutator to a differential amplifier (Bak Electronics), which bandpass filtered between 500 Hz and 5 kHz. Acoustic activity was picked-up by a Sennheiser ME62/K6-C microphone. Auditory and neural signals were digitized (at 8 kHz and 20 kHz, respectively) and stored simultaneously using Discovery commercial software package from Datawave Technologies (Longmont, CO). Auditory signals were also recorded on a Sony DAT recorder for singing versus listening experiments. Conspecific songs not used for recording experiments were used as search stimuli for auditory sites. Once recording at a site was exhausted, either by testing of all parameters, or loss of auditory response, the electrode was advanced ventrally in approximately 0.04mm increments until a new auditory site was reached. Subjects remained connected for 4-8 weeks and were comfortable enough to sing both directed and undirected song after a few days.

Digitized Playback

Digitized BOS, rev BOS, conspecific song and canary song were presented as an interleaved set for 50 repetitions each at 10-second interstimulus intervals (order varied from experiment to experiment) through a speaker placed approximately 60cm from the recording chamber. Peak loudness of the stimuli ranged from 55-60dB (background 25dB, measured on the A-scale). Neuronal activity was continuously recorded in response to playback. The start of each stimulus presentation was marked in the raw neural data by hand. Where movement artifacts were detected, the trial was discarded. Movement in more than 25 out of 50 trials for a particular stimulus resulted in the data for that stimulus being discarded.

Singing versus Listening

Neuronal activity was continuously recorded while singing directed song towards a female, or undirected song for 50-70 bouts. Within 30 minutes after stopping recording, the bird's vocal output was played back through a speaker and the neural signal during passive listening was continuously recorded. Tape recorded song

was presented at 60-65dB peak loudness. Subjects sometimes countersang to playback. When countersinging coincided with stimulus presentation, or occurred within 500ms of the other neuronal responses during that period were not used. Each song bout was marked in the raw neural data by hand at the start of the first non-introductory note. Where movement artifacts were detected, the trial was discarded. As well, neural data for individual syllables was also marked by hand to overcome temporal jitter which occurs in natural song.

Data Analysis

Multiunit neural activity was analyzed on the fly for events occurring above a threshold set 1.5-2 times that of background. Events were represented in dot raster and histogram form. Events were counted in 10ms bins for both. For dot raster analysis, time is shown along the x-axis and trial number along the y-axis. Trial number increases from bottom to top and individual counts are shown as single dots. Histogram analysis displays time on the x-axis, and the total number of counts within a 10ms bin along the y-

axis. Background activity was measured from -1000ms to -500ms before the start of song being careful to ensure no sound was present during the singing versus listening experiments.

For digitized playback analysis, the average MUA was calculated by dividing the mean number of events detected during stimulus playback by the stimulus duration \pm standard error.

Silent periods in the stimuli of duration >40 ms were not included.

For singing versus listening data, the average MUA measured the mean number of events that occurred for the specified period \pm standard error.

The habituation ratio was devised as a measure of the degree to which activity declined over the course of the experiment for a particular stimulus. It was calculated as $[1 - (\frac{\text{the mean number of events for the last five presentations}}{\text{the mean number of events for the first five presentations}})]$. A habituation ratio near zero indicated that activity changed very little, while a value close to one was indicative of a large decrease in activity. Activity was not observed to increase in response to playback. All habituation ratio values were normalized to background which was counted as zero.

Histology

After recording for 4-8 weeks, birds were deeply anesthetized with Nembutal (25 μ l at 50mg/ml) and electrolytic lesions were made at, and above the recording sites. Animals were perfused transcardially with 0.9% saline followed by 3% formaldehyde. The brains were then sectioned by vibratome at 40 μ m thickness and cresyl stained to establish the position of recording sites within NCM.

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