EVOLUTIONARY AND FUNCTIONAL NEUROBIOLOGY OF BIRDSONG

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Birdsong is a complex, learned vocal communication signal that functions in reproduction. Many aspects of the neural mechanisms underlying this behavior remain unknown, with respect to both song production and perception. The work described here investigates these issues from different levels of analysis. Nearly every aspect of song is characterized by immense interspecific diversity. In particular, species range from those that learn a single syllable to others that produce hundreds or thousands, but neither the ultimate nor proximate causes for this variability have been identified. Chapter 2 uses comparative analyses across a wide phylogeny of songbirds to address these questions. Syllable repertoire size is positively and strongly correlated with the degree of convergence along the descending motor pathway, suggesting that the level of top-down control rather than motor circuit size *per se* is more closely associated with the complexity of behavioral output. Repertoire size is not related to the sizes of other brain regions, however. Chapter 3 describes general evolutionary patterns of functional neural circuits and tests predictions made by two models of brain evolution. Interestingly, support was found for both. Nuclei that develop late in ontogeny possess larger allometric slopes than those that develop early, which is consistent with a developmental conservation model in which a general stretching of neurogenetic schedules underlies increases in brain size. Functional circuits evolve in concert and do so independently of others, however, supporting a mosaic pattern. Finally, chapter 4 examines the function of the caudomedial

nidopallium (NCM), a region of the auditory forebrain, in adult females. Numerous correlative studies suggest that NCM is important for song learning. Consistent with this, inactivation of NCM eliminates female preferences for familiar songs but has much more modest effects on their preferences for high quality songs. Together, these experiments are the first to relate evolutionary changes in behavioral capacities to those in its underlying circuit, to document the coordinated evolution of functional circuits, and to demonstrate a causative role for the auditory forebrain in song perception.

BIOGRAPHICAL SKETCH

Jordan Moore hails from the small town of Waterloo, Wisconsin, where the cows outnumber the people. It was here that Jordan developed a deep appreciation for the finer things in life, including the Green Bay Packers, bratwursts, and deep-fried cheese curds. Jordan graduated from Waterloo High School in 1998 and attended the University of Wisconsin-Madison, where he majored in biology and zoology. Unofficially, he also majored in making plays. Jordan had many great experiences at the University of Wisconsin. Among them, he gained his first research experience in the lab of Prof. Tom Yin working with Dr. Dan Tollin. These two were terrific mentors and this positive experience influenced Jordan to pursue a career in neuroscience. Before beginning graduate school, however, Jordan wanted to experience life outside the Midwest and moved to Salamanca, Spain for a year. He then came to Ithaca, a small, dreary town in New York. While not particularly fond of his new surroundings, he nevertheless met some great friends and good times were had. He fondly recalls Jon's matchbox car, Damian playing sheepshead, golfing with Dave, the Rio trio, the lost week with Chuck crashing on the couch, Matt going balls to the wall, and of course Cakes' Friday Night Lights. Six long years later, the curtain falls.

To mom and dad

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CHAPTER 1

INTRODUCTION

Songbirds have long been admired their varied and complex songs, and a great deal of research has sought to fully describe this behavior with respect to each of Tingbergen's (1963) four questions: its function, evolution, development, and underlying neural mechanisms. Birdsong is a learned vocal communication signal that functions in reproduction and is generally thought to have evolved in response to sexual selection. Typically, males sing to attract females as mates and/or defend territories against competing males. The song features under selection can differ between taxonomic groups, but large syllable repertoires are a favored phenotype in several. Females of many species prefer to mate with males that possess larger repertoires (Byers & Kroodsma 2009), and repertoires must somehow be costly to senders to have evolved as honest signals of male quality (Zahavi 1975a; Bradbury & Vehrencamp 1998). Within species, repertoire size reflects developmental history: males that experience nutritional or immunological stresses early in life develop smaller repertoires (Nowicki et al. 1998; Nowicki & Searcy 2004), and this association can persist throughout life as adult male song sparrows (*Melospiza melodia*) with larger repertoires have superior immune systems and are in better physiological condition (Pfaff et al. 2007). Repertoires may also signal general properties of the brain. Song learning and production are controlled by a discrete, evolutionarily conserved neural circuit dedicated specifically to this purpose. Within species, the size of the premotor nucleus HVC is heritable, is positively related to song complexity, and is correlated with overall brain size (Airey et al. 2000; Garamszegi & Eens 2004). The research described here further characterizes some of these relationships from different levels of analysis, focusing on the coordinated evolution

between song complexity and its underlying neural circuit, the evolution of the song system with respect to other neural circuits, and the neural pathways underlying song perception in females.

Songbirds are remarkable among the animal kingdom because they learn a large portion of their vocal repertoire. While they are not unique in this regard, they are by far the largest group of animals possessing this capacity with each of the approximately 4000 species learning its own characteristic song. Species can vary in nearly every aspect of the learning process, including the accuracy with which they copy tutor songs, their ability for improvisation, the types of sounds they attempt to copy, the time of life during which memorization and vocal modification occurs, and the social interactions necessary and/or sufficient to produce normal learning. This immense diversity is perhaps best exemplified by interspecific differences in the capacity to learn multiple song components, or syllables, with species ranging from those that learn a single syllable to others than produce hundreds or thousands. Moreover, song is controlled by a conserved network of interconnected nuclei dedicated specifically to this purpose. Together, these attributes make songbirds ideally suited for comparative analyses to help discover principles of motor circuit organization because differences in behavior can be directly related to those in the structure of its underlying pathway.

Previous studies have identified positive relationships between repertoire size and the volume of nucleus HVC (e.g., DeVoogd et al. 1993; Garamszegi & Eens 2004). These anatomical correlates are consistent with physiological data showing that HVC neurons sparsely encode song, whereby each individual cell only fires during a single, brief segment of song (Hahnloser et al. 2002). Thus, increases in repertoire appear to require the addition of HVC neurons to encode this increase in vocal versatility. However, these correlations are often quite weak and HVC is not

singly responsible for song production. Chapter 2 explores this brain-behavior relationship in greater depth by relating syllable repertoire size to the structure of the entire song circuit. Surprisingly, the degree of convergence along this motor pathway is more closely related to repertoire size than its overall size, suggesting that the level of top-down control could be a primary determinant of behavioral complexity. This chapter also seeks to identify potential functions of repertoires by searching for evolutionary correlations between repertoire size and the relative volumes of structures not directly involved in song production. No such relationships are evident, however, suggesting that repertoires have not evolved as reliable signals for general cognitive capacities.

A related issue concerns the evolution of the brain in general. Two models have been proposed to describe changes in brain composition as its overall size changes. The first, referred to as the model of developmental conservation, suggests that neurogenetic schedules are largely conserved but stretched or shortened in duration and differences in composition between large and small brains are largely predictable from their developmental sequence (Finlay & Darlington 1995). This model specifically predicts that late-developing structures will become disproportionately large as overall size increases owing to exponential increases in the number of neuronal precursors. The second model, referred to as mosaic evolution, focuses more on the relationships between areas and predicts that those sharing functional connections will evolve in concert but will be independent of structures underlying other functions (Barton & Harvey 2000). While not mutually exclusive, these ideas are often portrayed as such and several reports have sought to falsify one model by providing support for the other (e.g., de Winter & Oxnard 2001). Furthermore, nearly all work in this area has focused on mammals and all analyses have been limited to the sizes of major brain subdivisions that contain areas belonging

to many distinct functional systems. Chapter 3 addresses some of these limitations by documenting the evolution of discrete neural circuits in songbirds, in which the pallia are organized into nuclei rather than layers which permits the direct and accurate measurement of areas belonging to various functional systems. Interestingly, elements of both models are supported. Telencephalic nuclei incorporate neurons late in ontogeny and exhibit greater allometric slopes than areas in the thalamus and brainstem, which cease neuronal recruitment at earlier times. The relative volumes of nuclei within one system are also strongly related to each other, regardless of brain subdivision, and are largely independent of structures belonging to a different system. Finally, the song system develops late compared to surrounding tissue and is much more variable than nuclei within other systems, perhaps facilitating the extreme variability of the song system compared to other systems and the immense behavioral diversity between species.

Lastly, female song preferences drive many evolutionary changes in male song and thus in underlying brain structure, but much remains unknown about the nature of these preferences. In particular, the neural substrate underlying song perception has only recently been identified and the specific functions of different regions are unclear. One area that has received special attention is the caudomedial nidopallium (NCM) because this area responds selectively to song and its physiological and genomic responses habituate to repeated stimulus presentations, suggesting that it may play an important role in song memory formation and/or storage (Mello et al. 1992, 1995; Chew et al. 1995). Yet, despite the many studies that have characterized responses in this area following presentation of various auditory stimuli, none have demonstrated its causal role in song perception. Chapter 4 assesses the role of NCM in song perception directly in female zebra finches (*Taeniopygia guttata*). Using a phonotaxis task, the role of NCM in song perception is measured for two distinct types

of song preferences shown by adult females. The first is based on song familiarity; females prefer the songs of their mate or their father over unfamiliar songs (Miller 1979a,b). The second is based on song quality and is independent of previous song exposure; females prefer longer songs composed of more syllables and also prefer normal, learned songs to improvised songs that have abnormally simple phonology (Neubauer 1999b; Lauay et al. 2004). Inactivation of NCM eliminated female preferences for their mate's song but had much more modest effects on those for high quality songs, indicating that NCM is necessary for preferences based on song familiarity but not those based on song quality.

In conclusion, the work presented here contributes to new areas of birdsong research from different levels of analysis. It is the first to relate evolutionary changes in behavioral capacities to the structure of its underlying neural circuit. It is the first to document the evolution of discrete neural circuits. It is also the first to demonstrate that an area of the auditory forebrain is required for a specific type of song preference. These findings provide a foundation for future work, such as investigations into the evolution of neuronal morphologies within song nuclei, developmental mechanisms that underlie coordinated changes between nuclei, and the neural pathways that produce the expression of distinct song preferences by female songbirds.

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CHAPTER 2

DEGREE OF CONVERGENCE ALONG A MOTOR PATHWAY IS STRONGLY RELATED TO BEHAVIORAL COMPLEXITY ACROSS A WIDE PHYLOGENY OF SONGBIRDS

Abstract

Songbirds have long been renowned for their varied and complex songs, which are learned vocal communication signals that have been shaped by sexual selection. Species especially differ in their capacity for learning multiple song components, but much remains unknown about the proximate and ultimate bases for such variety. This comparative study describes evolutionary correlations between syllable repertoires and the structure of the song system across a wide phylogeny of mostly unstudied songbird species. The relative volumes of song nuclei evolved in concert but can differ markedly in their variability about allometric expectations. In particular, the premotor nucleus HVC and one of its primary afferents, the medial magnocellular nucleus of the anterior nidopallium (MMAN), are much more variable than other areas. Both relative HVC and MMAN volumes are positively correlated with repertoire size. Relationships were substantially improved, however, when nucleus volumes were considered relative to their efferent target(s) rather than overall size. These findings suggest that evolutionary changes in repertoire size have been more closely associated with changes in the degree of convergence along this motor pathway than relative circuit size *per se*.

Introduction

Birdsong is a complex, learned vocal communication signal that functions in reproduction. It is an incredibly diverse behavior, with each of the approximately 4000 oscine species producing characteristic song(s) that can differ in nearly every

respect. This variability is perhaps best exemplified by repertoires; species range from those that learn only a single song component, or syllable (e.g., chipping sparrow, *Spizella passerina*; Liu & Kroodsma 1999) to others that produce hundreds of distinct sounds (e.g., brown thrasher, *Toxostoma rufum*; Kroodsma & Parker 1977). Such immense disparities in behavioral capacities are seldom seen within so large a group of closely related species, yet it remains unknown how or why such large differences exist.

Song learning and production are primarily controlled by the song system, a discrete and evolutionarily conserved neural circuit dedicated specifically to this purpose (Figure 2.1; Nottebohm et al. 1976; 1982). This network is largely comprised of two parallel pathways arising from sensorimotor nucleus HVC: a caudal motor pathway underlies song production (Nottebohm et al. 1976; Vu et al. 1994), and a rostral, basal ganglia pathway generates motor variability and is required for song learning but not for stable song production in adults (Bottjer et al. 1984; Scharff & Nottebohm 1991; Kao et al. 2005; Ölveczky et al. 2005). The discrete nature and functional specificity of this circuit, coupled with its quantifiable output, make songbirds well suited for comparative analyses that can address the evolution, function, and proximate causes of a complex motor behavior.

The neural bases underlying the capacity for complex songs have not been unequivocally identified, but three lines of evidence suggest that HVC size is a primary determinant of this ability. First, repertoire size is positively correlated with HVC volume within species (Nottebohm et al. 1981; Garamszegi & Eens 2004), between species (DeVoogd et al. 1993; Székely et al. 1996), and between sexes (Brenowitz et al. 1985; MacDougall-Shackleton & Ball 1999). HVC volume is largely independent of early auditory experience, therefore increases in HVC neuron number appear to be permissive for more learning rather than a consequence of it

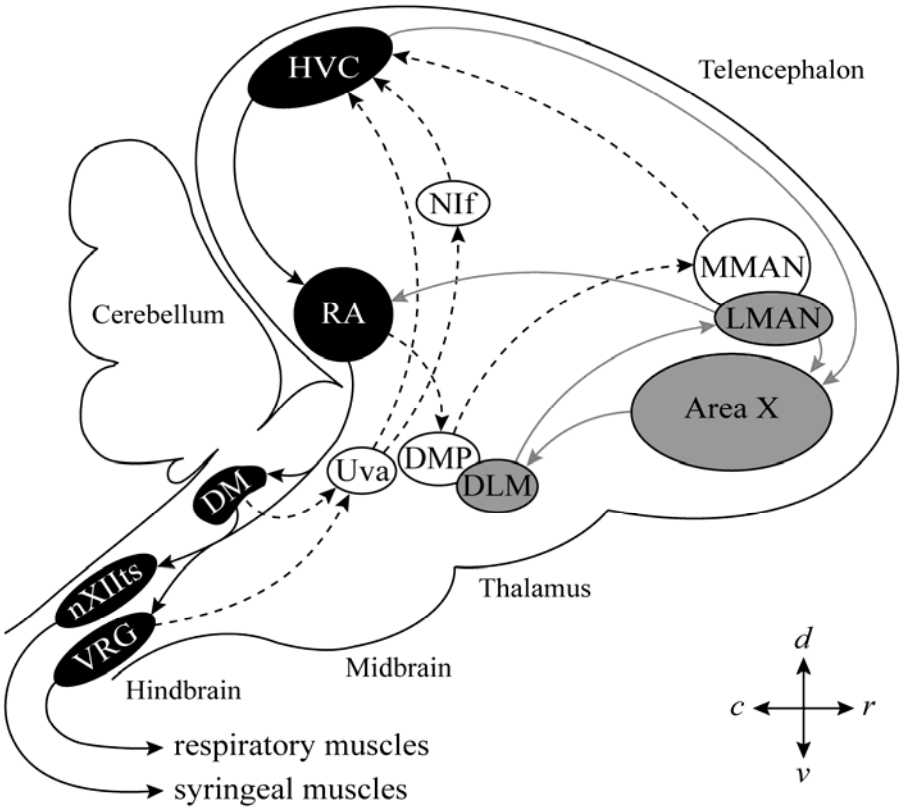


Figure 2.1. Diagram of the song system. The caudal motor pathway is shown in black, the anterior forebrain pathway in gray, and feedback projections with dashed lines and open circles. DMP: dorsomedial nucleus of the posterior thalamus; DLM: dorsolateral nucleus of the lateral anterior thalamus; VRG: ventral respiratory group.

(Brenowitz et al. 1995; Leitner et al. 2002). Second, song complexity and the size of HVC covary after various experimental treatments, such as nutritional or immunological stresses during development (Spencer et al. 2005; Pfaff et al. 2007) or administration of endocrine-disrupting chemicals (Markman et al. 2008). Third, intricate physiological studies have shown that HVC encodes song with a sparse code, whereby individual neurons that project to the robust nucleus of the arcopallium (RA) fire a temporally precise burst of action potentials that corresponds to one specific syllable segment during each song rendition (Hahnloser et al. 2002). If such specificity is general across songbirds, and it appears to be (Mooney et al. 2001; Prather et al. 2008), then an ability to produce a larger repertoire would seem to necessitate more HVC neurons.

Nevertheless, the relationship between HVC and repertoire size remains contentious because several inconsistencies among studies appear to contradict notions of a strict correspondence between the two. The majority of intraspecific studies have not detected significant correlations (e.g., Kirn et al. 1989; Brenowitz et al. 1991); there are sizeable outliers from the parallel sex differences in song behavior and anatomical dimorphisms (Gahr et al. 1998, 2008); age-related increases in repertoire are not necessarily accompanied by expansions of HVC (Nottebohm et al. 1986); and seasonal fluctuations in HVC volume are not accompanied by changes in repertoire (Kirn et al. 1989; Brenowitz et al. 1991; Smith et al. 1995). These observations call attention to the seemingly unreliable nature of the HVC and song complexity relationship.

Comparative analyses can also provide clues about potential behavioral function(s). Birdsong has evolved in the context of sexual selection, where males typically sing to attract mates and/or defend territories and females assess song to gauge male quality. Females of many species prefer large repertoires, which can

convey direct and/or indirect benefits to be gained (Buchanan & Catchpole 2000; Nowicki & Searcy 2004). Regarding the latter, intraspecific studies have shown that male repertoires can honestly signal genetic diversity, developmental history, physiological condition, immunocompetence, parasite load, and overall viability (e.g., Hasselquist et al. 1996; Nowicki et al. 1998; Marshall et al. 2003; Buchanan et al. 2004; Pfaff et al. 2007). By virtue of their relation to HVC, repertoires may also indicate features about the brain in general. Such a function was suggested by the findings that HVC volume is heritable and is related to the sizes of other song nuclei and the telencephalon (Burek et al. 1991; Airey et al. 2000).

The goals of this study were threefold: (1) to re-examine the evolutionary relationship between syllable repertoire size and song system anatomy using a new data set of mostly unstudied species; (2) to describe general evolutionary patterns of the song circuit, and (3) to test for correlations between repertoire and brain regions not directly involved in song. We find that consideration of multiple song nuclei rather than HVC alone greatly enhances the correlation with behavior.

Methods

(a) *Specimen collection and preparation*

All procedures were approved by the Cornell University Institutional Animal Care and Use Committee. One to four adult male songbirds of 58 temperate zone species were wild-caught under permit with mist nets. Collections were restricted to spring months (April – June) when birds were reproductively active to minimize seasonal variation in song system anatomy. Most were collected throughout Hungary from 1993-1995 or in Tompkins County, New York in 2004. Exceptions are the white-throated sparrows (*Zonotrichia albicollis*), Ontario, Canada in 1991; European pied flycatchers (*Ficedula hypoleuca*), central Norway in 1995; yellow-throated buntings (*Emberiza elegans*), China in 1991; and northern mockingbirds (*Mimus*

polyglottos), North Carolina in 2003. Birds were deeply anesthetized with barbiturate anesthetic at the time of capture and transcardially perfused with 0.8% saline followed by 10% formalin in saline. Brains were extracted, post-fixed for at least 24 hours, cryoprotected with 30% sucrose/10% formalin, embedded in gelatin, and sectioned at 40 μm in the coronal plane with a freezing, sliding microtome. Sections were then mounted onto gel-coated slides, Nissl-stained with cresyl violet, and coverslipped using Permount.

(b) *Brain measurements*

Alternate sections were viewed under 40× or, for larger regions [mesopallium (MP) and hippocampus (Hp)], every fourth section was viewed under 20× magnification and nucleus boundaries were traced using a camera lucida. Unmagnified digital images of every fourth section were used to measure the telencephalon. The song nuclei measured were HVC, RA, Area X of the striatum, the lateral and medial magnocellular nuclei of the anterior nidopallium (LMAN and MMAN, respectively), uvaeformis (Uva), the dorsomedial nucleus of the intercollicular complex (DM), and the tracheosyringeal portion of the hypoglossal nucleus (nXIIts). Other brain regions included MP, three limbic structures [Hp, septum (Sep), and nucleus taeniae of the amygdala (TnA)], and an auditory nucleus [dorsal part of the lateral mesencephalic nucleus (MLd)]. Measurements were made in one side of the brain, typically the left except in cases where torn tissue or poor staining prevented measurements. Cross-sectional areas of scanned traces and telencephalon images were measured using NIH ImageJ software (Rasband 2007) and final volumes were computed by summing the areas and multiplying by the sampling interval (0.08 or 0.16 mm).

The boundaries of most song nuclei were unambiguous. Song control nucleus HVC, however, is complex and comprised of at least three distinct subdivisions

(Fortune & Margoliash 1995). Identified boundaries were based on two that are visible in the coronal plane: a central region that contains many large, darkly-stained cells and the caudomedial region or paraHVC, which is comprised of small, densely-packed neurons. In most cases, MMAN and LMAN were not contiguous and were easily distinguished. When the two were juxtaposed, MMAN was delineated on the basis of its slightly smaller somata and generally higher cell density than LMAN. The nucleus interface of the nidopallium (NIf) could not be reliably identified in many specimens and the medial portion of the dorsolateral nucleus of the anterior thalamus (DLM) lacks clear boundaries, therefore neither was measured.

Rostral MP was identified by differences in staining intensity compared to adjacent regions; in more central and caudal sections, it was clearly distinguished by the superior frontal and mesopallial laminae. The lateral extent of Hp was identified by its lower cell density compared to adjacent areas (Székely & Krebs 1996). Caudal Hp does not have a clear boundary with the surrounding parahippocampal area, therefore measurements were arbitrarily stopped at the section in which the cerebellum reached the dorsal-most extent of the telencephalon. Sep boundaries were identified by differences in staining intensity and largely coincided with those described by chemoarchitecture (Goodson et al. 2004); the estimates reported here include most of the four major subdivisions but exclude portions of the nucleus of the diagonal band ventral to the septopallio-mesencephalic tract. Both TnA and MLd were delineated on the basis of their staining intensity and/or cell density.

Neuronal densities in HVC, RA, and nXIIts were estimated from one brain of each species. Presumptive neurons were discriminated from glia on the basis of their soma size, uniformly stained cytoplasm, and a single, darkly stained nucleolus. Nucleolus counts were tallied within sampling windows that were evenly distributed throughout each plane of each structure, and a neuron was only counted if its

nucleolus was within the grid. Grid dimensions were 80 × 80 μm (600×, HVC and nXIIts) or 120 × 120 μm (400×, RA). On average, 13 (range: 7-26) counts were made in each nucleus.

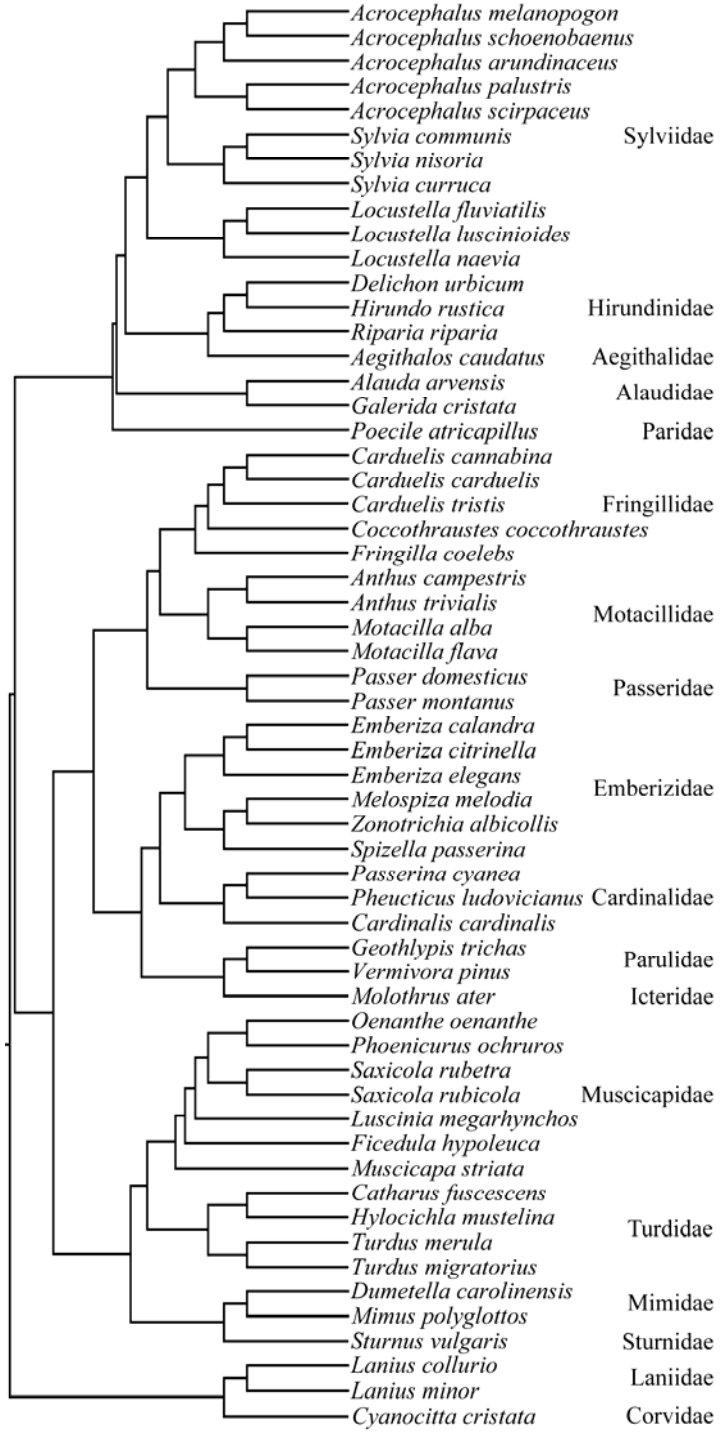
(c) *Syllable repertoires*

The behavioral unit of interest was a song syllable. A syllable is generally defined as a continuous trace on a spectrogram or a stereotyped sequence of notes separated by less than 25 msec, and a repertoire is the number of unique syllables produced by an individual bird. Species-typical syllable repertoires were obtained for 49 of the 58 species, most from published sources. When multiple sources were found for a species, one was chosen on the basis of sample size, clarity of the syllable definition and conformance to that above, proximity of the recording site to the site of specimen collection, and the season during which recordings were made. Repertoires for seven species were estimated from recordings held by the Macaulay Library at Cornell University (mean: 3 birds per species, range: 1-5) with the Syrinx sound analysis program (Burt 2006). All recorded syllables known to originate from one individual (mean: 199 syllables per bird, range: 14-528) were used to construct plots of new syllables encountered versus total syllables sampled. The function $y = a(1 - e^{-bx})$ was fit to the data and the asymptote (a) was used as the estimated repertoire.

(d) *Phylogeny construction*

A fully resolved, composite phylogeny was constructed from published molecular phylogenies (Figure 2.2). Species spanned 18 families, 16 of which are within the Passerida parvorder. All inter-family relationships were inferred from the analysis of two nuclear genes (RAG-1 and RAG-2). Most intra-family relationships were based on the mitochondrial gene(s) cytochrome *b* and/or ND2, occasionally in

Figure 2.2. Composite phylogeny of 58 species used in this study. Branch lengths were scaled using the method of Grafen (1989) and the rho transform ($\rho = 0.3$). Sources were: inter-family (Barker et al. 2004); Sylviidae (Blondel et al. 1996; Leisler et al. 1997; Helbig & Seibold 1999; Drovetski et al. 2004); Hirundinidae (Sheldon et al. 2005); Fringillidae (Arnaiz-Villena et al. 2001; Klicka et al. 2003; Zamora et al. 2006); Motacillidae (Arctander et al. 1996); Emberizidae (Klicka et al. 2007; Alström et al. 2008); Cardinalidae (Yuri & Mindell 2002); Muscicapidae (Wink et al. 2002; Voelker & Spellman 2004); Turdidae (Klicka et al. 2005); Mimidae (Cibois & Cracraft 2004).


Acrocephalus melanopogon
Acrocephalus schoenobaenus
Acrocephalus arundinaceus
Acrocephalus palustris
Acrocephalus scirpaceus
Sylvia communis
Sylvia nisoria
Sylvia curruca
Locustella fluviatilis
Locustella luscinioides
Locustella naevia
Delichon urbicum
Hirundo rustica
Riparia riparia
Aegithalos caudatus
Alauda arvensis
Galerida cristata
Poecile atricapillus
Carduelis cannabina
Carduelis carduelis
Carduelis tristis
Coccothraustes coccothraustes
Fringilla coelebs
Anthus campestris
Anthus trivialis
Motacilla alba
Motacilla flava
Passer domesticus
Passer montanus
Emberiza calandra
Emberiza citrinella
Emberiza elegans
Melospiza melodia
Zonotrichia albicollis
Spizella passerina
Passerina cyanea
Pheucticus ludovicianus
Cardinalis cardinalis
Geothlypis trichas
Vermivora pinus
Molothrus ater
Oenanthe oenanthe
Phoenicurus ochruros
Saxicola rubetra
Saxicola rubicola
Luscinia megarhynchos
Ficedula hypoleuca
Muscicapa striata
Catharus fuscescens
Hylocichla mustelina
Turdus merula
Turdus migratorius
Dumetella carolinensis
Mimus polyglottos
Sturnus vulgaris
Lanius collurio
Lanius minor
Cyanocitta cristata
Sylviidae
Hirundinidae
Aegithalidae
Alaudidae
Paridae
Fringillidae
Motacillidae
Passeridae
Emberizidae
Cardinalidae
Parulidae
Icteridae
Muscicapidae
Turdidae
Mimidae
Sturnidae
Laniidae
Corvidae

addition to a nuclear gene; two exceptions were the Mimidae family (nuclear RAG-1) and the *Sylvia* genus (nuclear DNA hybridization).

(e) *Data analysis*

Specimens representing 13 of the 58 species in this data set were the subjects of previous studies (DeVoogd et al. 1993; 1995; Székely et al. 1996). All areas were re-measured, and exclusion of these data did not alter the significance of principal findings reported here. Mesquite (Maddison & Maddison 2008) was used to manage data and trees, and the PDAP:PDTree v1.14 module was used for all analyses of independent contrasts (Garland et al. 1999; Garland & Ives 2000). Phylogenetic signal was computed with the Matlab program PHYSIGLL.m (Blomberg et al. 2003) and phylogenetic generalized least squares (PGLS) models were constructed with REGRESSIONv2.m (Lavin et al. 2008). For these analyses, trees were first converted to variance-covariance matrices with the PDDIST module of Phenotypic Diversity Analysis Programs (Garland & Ives 2000).

PGLS models using $\log_{10}$-transformed data were used for most analyses. Arbitrary branch lengths computed with the method of Grafen (1989) and scaled with the rho ($\rho = 0.3$) transform were used because of this tree's consistent performance in diagnostic tests for all traits (Garland et al. 1992; Blomberg et al. 2003). For comparison, data were also analyzed using a star phylogeny (a single basal polytomy with equal branch lengths, as assumed by conventional statistics). Models were constructed to determine relationships between song nuclei and between syllable repertoire and various combinations of nuclei; in each case, a 'size' parameter was included as a covariate. Values used for the 'size' reference depended on the trait(s) in question and/or the particular analysis. For song nuclei, limbic structures, and MP it was the difference between the telencephalon and respective total circuit volume (i.e. sum of song nuclei or limbic structures) or subdivision (i.e. MP) to control for

part-whole artifacts. For MLd, it was telencephalon volume and for the telencephalon it was species-typical body mass (Dunning 2008). Residual analyses were also performed in some cases, in which relative trait sizes were first computed and then regressed with another trait (Darlington & Smulders 2001). Here, allometric slopes of $\log_{10}$-transformed song nucleus volumes were estimated using either the same 'size' reference listed above (e.g., HVC versus telencephalon) or another nucleus (e.g., HVC versus RA). These were then used to compute relative trait volumes with the formula $\log_{10}[\text{trait}/(\text{size}^b)]$, where b was the allometric exponent, and 'trait' and 'size' were original data values. Data from all 58 species were used for brain-only analyses, but only the 49 for which repertoires were obtained were used in the brain-behavior correlations. Prior to the latter analyses, the phylogenetic tree was pruned, branch lengths re-scaled and diagnostic tests re-run. Nested and non-nested models were compared using ln maximum likelihood ratio tests (LRTs) and the Akaike Information Criterion (AIC; smaller is better), respectively, and partial F-tests were used to test for significance of independent variables in multivariate models. Note that results from PGLS models and independent contrasts are identical (Garland & Ives 2000), and R^2 values from PGLS models are not comparable to those from traditional ordinary least squares models.

Results

Species-typical repertoires and their respective sources are listed in Table 2.1. The use of different branch lengths produced qualitatively consistent results, therefore only those based on the hierarchical tree are presented here.

(a) *Allometry of song nuclei*

All absolute and most relative nucleus volumes exhibited significant phylogenetic signal, indicating that they were better described by the hierarchical than star phylogeny under a Brownian motion model of evolution (Table 2.2). The

Table 2.1. Syllable repertoires and sources used in this study. Common names follow Gill & Wright (2006).

Common Name	Scientific Name	Syl Rep	Source
American Goldfinch	*Carduelis tristis*	33	Macaulay Library (*n* = 5)
American Robin	*Turdus migratorius*	38	Dobson & Lemon (1979)
Barn Swallow	*Hirundo rustica*	20	Garamszegi et al. (2005)
Barred Warbler	*Sylvia nisoria*	74	Székely et al. (1996)
Black Redstart	*Phoenicurus ochruros*	5	Macaulay Library (*n* = 1)
Black-capped Chickadee	*Poecile atricapillus*	2	Kroodsma et al. (1999)
Blue Jay	*Cyanocitta cristata*		
Blue-winged Warbler	*Vermivora pinus*	4	Gill & Murray (1972)
Brown-headed Cowbird	*Molothrus ater*	15	Allan & Suthers (1994)
Chipping Sparrow	*Spizella passerina*	1	Albrecht & Oring (1995)
Common Blackbird	*Turdus merula*	108	Garamszegi et al. (2005)
Common Chaffinch	*Fringilla coelebs*	18	Garamszegi et al. (2005)
Common Grasshopper Warbler	*Locustella naevia*	1	Wahlström (1966)
Common House Martin	*Delichon urbicum*		
Common Linnet	*Carduelis cannabina*		
Common Nightingale	*Luscinia megarhynchos*	1160	Hultsch (1980)
Common Starling	*Sturnus vulgaris*	44	Garamszegi et al. (2005)
Common Whitethroat	*Sylvia communis*	119	Garamszegi et al. (2005)
Common Yellowthroat	*Geothlypis trichas*	5	Lemon et al. (1983)
Corn Bunting	*Emberiza calandra*	9	Macaulay Library (*n* = 2)
Crested Lark	*Galerida cristata*	55	Macaulay Library (*n* = 1)
Eurasian Reed Warbler	*Acrocephalus scirpaceus*	80	Catchpole (1980)
Eurasian Skylark	*Alauda arvensis*	341	Briefer et al. (2008)
Eurasian Tree Sparrow	*Passer montanus*	10	Lang & Barlow (1997)
European Goldfinch	*Carduelis carduelis*	60	Güttinger & Clauss (1982)

Table 2.1 (Continued).

Common Name	Scientific Name	Syl Rep	Source
European Pied Flycatcher	*Ficedula hypoleuca*	27	Espmark & Lampe (1993)
European Stonechat	*Saxicola rubicola*	93	Schwager & Güttinger (1984)
Great Reed Warbler	*Acrocephalus arundinaceus*	30	Forstmeier et al. (2006)
Grey Catbird	*Dumetella carolinensis*	328	Kroodsma et al. (1997)
Hawfinch	*Coccothraustes coccothraustes*		
House Sparrow	*Passer domesticus*	6	Nivison (1978)
Indigo Bunting	*Passerina cyanea*	8	Payne (1981)
Lesser Grey Shrike	*Lanius minor*		
Lesser Whitethroat	*Sylvia curruca*	68	Klit (1999)
Long-tailed Bushtit	*Aegithalos caudatus*		
Marsh Warbler	*Acrocephalus palustris*	412	Bell et al. (2004)
Moustached Warbler	*Acrocephalus melanopogon*	134	Feßl & Hoi (2000)
Northern Cardinal	*Cardinalis cardinalis*	14	Anderson & Conner (1985)
Northern Mockingbird	*Mimus polyglottos*	216	Wildenthal (1965)
Northern Wheatear	*Oenanthe oenanthe*	46	Macaulay Library (n = 5)
Red-backed Shrike	*Lanius collurio*		
River Warbler	*Locustella fluviatilis*	2	Wahlström (1966)
Rose-breasted Grosbeak	*Pheucticus ludovicianus*	19	Lemon & Chatfield (1973)
Sand Martin	*Riparia riparia*	3	Macaulay Library (n = 3)
Savi's Warbler	*Locustella luscinioides*	1	Wahlström (1966)
Sedge Warbler	*Acrocephalus schoenobaenus*	68	Buchanan & Catchpole (2000)
Song Sparrow	*Melospiza melodia*	38	Borror (1965)
Spotted Flycatcher	*Muscicapa striata*		

Table 2.1 (Continued).

Common Name	Scientific Name	Syl Rep	Source
Tawny Pipit	*Anthus campestris*	1	Wallschläger (1964)
Tree Pipit	*Anthus trivialis*	11	Wallschläger (1964)
Veery	*Catharus fuscescens*	12	Dobson & Lemon (1979)
Western Yellow Wagtail	*Motacilla flava*	2	Ödeen & Björklund (2003)
Whinchat	*Saxicola rubetra*	130	Schwager & Güttinger (1984)
White Wagtail	*Motacilla alba*		
White-throated Sparrow	*Zonotrichia albicollis*	5	Falls & Kopachena (1994)
Wood Thrush	*Hylocichla mustelina*	16	Dobson & Lemon (1979)
Yellowhammer	*Emberiza citrinella*	5	Garmaszegi et al. (2005)
Yellow-throated Bunting	*Emberiza elegans*	109	Zeng et al. (2005)

Table 2.2. Phylogenetic signal of $\log_{10}$-transformed trait values. Tree branch lengths were scaled using the method of Grafen (1989) and the rho transform ($\rho = 0.3$). Refer to Blomberg et al. (2003) and Revell et al. (2008) for discussions of the *K*-statistic and phylogenetic signal.

Trait	df	MSE_0	MSE_{star}	MSE	obs. MSE_0/MSE	exp. MSE_0/MSE	*K*	p	ln lklhd	ln $lklhd_{star}$
log(Mass)	56	0.078	0.075	0.058	1.351	1.518	0.890	**<0.001**	0.801	-6.847
log(Telen)	56	0.057	0.053	0.041	1.373	1.518	0.904	**<0.001**	10.711	3.190
log(HVC)	56	0.118	0.117	0.087	1.360	1.518	0.896	**<0.001**	-10.832	-19.599
log(RA)	56	0.066	0.065	0.060	1.090	1.518	0.718	**0.004**	-0.379	-2.416
log(Area X)	56	0.082	0.079	0.064	1.288	1.518	0.848	**<0.001**	-1.978	-8.265
log(LMAN)	56	0.069	0.068	0.065	1.057	1.518	0.696	**0.001**	-2.644	-3.949
log(MMAN)	56	0.099	0.098	0.071	1.394	1.518	0.918	**<0.001**	-5.074	-14.509
log(Uva)	56	0.029	0.028	0.024	1.242	1.518	0.818	**<0.001**	26.834	21.483
log(DM)	56	0.024	0.023	0.022	1.082	1.518	0.713	**0.002**	28.916	27.685
log(nXIIts)	56	0.045	0.044	0.044	1.011	1.518	0.666	**0.010**	8.643	8.789
log(MP)	56	0.067	0.063	0.050	1.338	1.518	0.881	**<0.001**	4.940	-1.767
log(Hp)	56	0.052	0.047	0.040	1.313	1.518	0.865	**<0.001**	11.635	6.886
log(Sep)	56	0.039	0.035	0.030	1.292	1.518	0.851	**<0.001**	19.711	15.162
log(TnA)	56	0.046	0.043	0.028	1.636	1.518	1.078	**<0.001**	21.877	9.291
log(MLd)	56	0.026	0.025	0.024	1.091	1.518	0.718	**0.001**	26.683	25.109
log(syl rep)	47	0.592	0.592	0.445	1.331	1.436	0.927	**<0.001**	-49.196	-56.165
rel_HVC	47	0.084	0.084	0.058	1.445	1.436	1.006	**<0.001**	0.734	-8.283
rel_RA	47	0.029	0.029	0.027	1.075	1.436	0.748	**<0.001**	19.835	18.130
rel_nXIIts	47	0.022	0.022	0.025	0.881	1.436	0.613	0.117	21.253	24.455
HVC_rel_RA	47	0.044	0.044	0.032	1.367	1.436	0.952	**<0.001**	15.062	7.435
RA_rel_nXIIts	47	0.020	0.020	0.020	0.997	1.436	0.694	**0.013**	27.265	27.339

allometric slopes of song nuclei were related to the brain subdivision within which they are located, with telencephalic nuclei having consistently larger slopes (0.77 – 0.85) than thalamic and brainstem structures (0.63 – 0.67) (Mann-Whitney rank sum test, $p = 0.04$; Table 2.3). There were striking differences in the variability of some nuclei. HVC and MMAN had comparatively weak allometric relationships ($0.31 \leq r^2 \leq 0.34$), while RA, Area X, LMAN, and nXIIts varied more directly with telencephalon size ($0.40 \leq r^2 \leq 0.50$) and Uva and DM tightly corresponded to it ($0.72 \leq r^2 \leq 0.76$). These relationships were homoscedastic (all $p > 0.12$). The extreme variability of HVC and MMAN is further underscored by comparisons between similarly-sized species. For example, the spotted flycatcher (*Muscicapa striata*) has a slightly smaller telencephalon (84%) than the common yellowthroat (*Geothlypis trichas*) yet has 15- and 9-fold larger absolute HVC and MMAN volumes, respectively. By comparison, its RA, Area X, and LMAN volumes are 3-4 times as large.

(b) *Correlations between song nuclei*

The volumes of most song nuclei were positively and strongly correlated after accounting for overall telencephalon size (Table 2.4). The main exception to this was DM, which was not correlated with any nuclei but had nearly significant relationships with HVC, RA, and Uva ($0.08 \leq p \leq 0.14$). Associations were usually strongest between monosynaptically-connected nuclei, and these generally remained significant in multivariate GLS models that explained variation in one nucleus as a function of all others (Table 2.5). For example, the only two significant partial coefficients in a model explaining HVC variation were attributable to a primary afferent source (MMAN, $p = 4.3 \times 10^{-5}$) and efferent target (RA, $p = 5.0 \times 10^{-5}$). Most significant relationships in these models were positive; the two exceptions included Uva, which

Table 2.3. Allometric data for traits used in the present analysis. Tree branch lengths were scaled using the Grafen (1989) and the rho transform ($\rho = 0.3$). In all cases, df = 56. SS: sum of song nuclei, LimbS: sum of limbic structures.

Brain Subdivision	X	Y	*r*	p	Slope
	log(Body Mass)	log(Telen)	0.908	<1.00E-16	0.766
Telencephalon	log(Telen-SS)	log(HVC)	0.560	4.94E-06	0.810
	log(Telen-SS)	log(RA)	0.705	6.68E-10	0.852
	log(Telen-SS)	log(Area X)	0.685	3.08E-09	0.851
	log(Telen-SS)	log(LMAN)	0.631	1.12E-07	0.793
	log(Telen-SS)	log(MMAN)	0.586	1.33E-06	0.768
Diencephalon	log(Telen-SS)	log(Uva)	0.850	<1.00E-16	0.642
Mesencephalon	log(Telen-SS)	log(DM)	0.869	<1.00E-16	0.634
Rhombencephalon	log(Telen-SS)	log(nXIIts)	0.647	4.19E-08	0.669
Telencephalon	log(Telen-MP)	log(MP)	0.945	<1.00E-16	1.052
	log(Telen-LimbS)	log(Hp)	0.916	<1.00E-16	0.898
	log(Telen-LimbS)	log(Sep)	0.914	<1.00E-16	0.779
	log(Telen-LimbS)	log(TnA)	0.834	4.44E-16	0.685
Mesencephalon	log(Telen)	log(MLd)	0.759	5.07E-12	0.576

Table 2.4. Matrix of relationships between nuclei with log(T-SS) as a confounding variable in each regression. Statistics refer to the partial coefficients of the variables indicated. Shaded cells indicate directly connected nuclei. In all cases, df = 55.

		log(HVC)	log(RA)	log(Area X)	log(LMAN)	log(MMAN)	log(Uva)	log(DM)
log(RA)	*r*	**0.702**						
	p	**1.19E-09**						
log(Area X)	*r*	**0.678**	**0.698**					
	p	**6.65E-09**	**1.58E-09**					
log(LMAN)	*r*	**0.497**	**0.699**	**0.717**				
	p	**8.42E-05**	**1.44E-09**	**3.46E-10**				
log(MMAN)	*r*	**0.741**	**0.461**	**0.569**	**0.398**			
	p	**4.26E-11**	**3.09E-04**	**3.93E-06**	**0.002**			
log(Uva)	*r*	**0.325**	**0.446**	**0.461**	**0.432**	0.047		
	p	**0.014**	**4.99E-04**	**3.10E-04**	**0.001**	0.730		
log(DM)	*r*	0.222	0.197	0.190	0.101	0.141	0.237	
	p	0.097	0.142	0.157	0.456	0.297	0.076	
log(nXIIts)	*r*	**0.329**	**0.630**	**0.504**	**0.509**	0.176	0.122	0.163
	p	**0.013**	**1.48E-07**	**6.32E-05**	**5.29E-05**	0.191	0.364	0.224

Table 2.5. Parameters of multivariate GLS models describing relationships between song nuclei. Each column represents a separate model with the nucleus in the top row as the response variable and all others as explanatory variables. Shaded cells indicate directly connected nuclei. In all cases, df = 50.

		log(HVC)	log(RA)	log(Area X)	log(LMAN)
log(HVC)	coef.	--	**0.316**	0.179	-0.166
	F	--	**13.881**	2.735	1.527
	p	--	**4.96E-04**	0.104	0.222
log(RA)	coef.	**0.687**	--	-0.029	**0.457**
	F	**13.881**	--	0.031	**5.759**
	p	**4.96E-04**	--	0.862	**0.020**
log(Area X)	coef.	0.290	-0.021	--	**0.469**
	F	2.735	0.031	--	**8.518**
	p	0.104	0.862	--	**0.005**
log(LMAN)	coef.	-0.178	**0.226**	**0.310**	--
	F	1.527	**5.759**	**8.518**	--
	p	0.222	**0.020**	**0.005**	--
log(MMAN)	coef.	**0.497**	-0.020	0.185	0.094
	F	**20.099**	0.051	3.460	0.559
	p	**4.31E-05**	0.822	0.069	0.458
log(Uva)	coef.	-0.029	**0.348**	**0.428**	0.259
	F	0.012	**4.352**	**5.007**	1.126
	p	0.912	**0.042**	**0.030**	0.294
log(DM)	coef.	-0.084	-0.043	-0.101	-0.239
	F	0.136	0.079	0.328	1.227
	p	0.713	0.780	0.570	0.273
log(nXIIts)	coef.	-0.199	**0.382**	0.228	0.095
	F	1.607	**16.653**	3.574	0.385
	p	0.211	**1.61E-04**	0.064	0.538
intercept	coef.	0.055	0.620	1.381	-0.675
	F	0.023	7.314	45.026	4.045
	p	0.880	0.009	1.71E-08	0.050
	R^2	0.814	0.877	0.844	0.770

Table 2.5 (Continued).

		log(MMAN)	log(Uva)	log(DM)	log(nXIIts)
log(HVC)	coef.	**0.577**	-0.009	-0.033	-0.157
	F	**20.099**	0.012	0.136	1.607
	p	**4.31E-05**	0.912	0.713	0.211
log(RA)	coef.	-0.051	**0.230**	-0.037	**0.654**
	F	0.051	**4.352**	0.079	**16.653**
	p	0.822	**0.042**	0.780	**1.61E-04**
log(Area X)	coef.	0.349	**0.213**	-0.064	0.293
	F	3.460	**5.007**	0.328	3.574
	p	0.069	**0.030**	0.570	0.064
log(LMAN)	coef.	0.117	0.085	-0.100	0.081
	F	0.559	1.126	1.227	0.385
	p	0.458	0.294	0.273	0.538
log(MMAN)	coef.	--	**-0.141**	**0.172**	-0.129
	F	--	**4.092**	**4.832**	1.257
	p	--	**0.048**	**0.033**	0.268
log(Uva)	coef.	**-0.536**	--	**0.718**	**-0.486**
	F	**4.092**	--	**33.938**	**5.035**
	p	**0.048**	--	**4.08E-07**	**0.029**
log(DM)	coef.	**0.512**	**0.563**	--	**0.448**
	F	**4.832**	**33.938**	--	**5.495**
	p	**0.033**	**4.08E-07**	--	**0.023**
log(nXIIts)	coef.	-0.190	**-0.188**	**0.221**	--
	F	1.257	**5.035**	**5.495**	--
	p	0.268	**0.029**	**0.023**	--
intercept	coef.	-1.159	-0.751	0.041	-1.201
	F	10.757	19.668	0.033	19.387
	p	0.002	5.06E-05	0.858	5.62E-05
	R^2	0.736	0.791	0.714	0.712

was inversely related to MMAN ($p = 0.048$) and nXIIts ($p = 0.029$) after accounting for the rest of the song circuit.

The cellular basis for some of these relationships was assessed by estimating neuron densities and numbers in HVC, RA, and nXIIts (Table 2.6). Neuron densities in each area were inversely related to telencephalon volume (all $p < 8.1 \times 10^{-9}$). After accounting for overall size, the volumes of all three nuclei were positively related to neuron number ($0.88 \leq r \leq 0.98$; all $p < 1.3 \times 10^{-19}$), but only those of RA ($r = -0.46$, $p = 2.7 \times 10^{-4}$) and nXIIts ($r = -0.56$, $p = 6.1 \times 10^{-6}$) were also inversely related to neuron density. Relative neuron numbers in each of the three nuclei were positively correlated ($0.31 \leq r \leq 0.58$; all $p < 0.02$), and those in HVC and RA were inversely related to neuron density in RA and nXIIts, respectively ($-0.46 \leq r \leq -0.42$; both $p < 0.001$).

(c) *Syllable repertoire size and song system neuroanatomy*

Initial analyses sought to explain variation in syllable repertoire size as a function of individual song nucleus volumes with size (T-SS) as a confounding variable (Table 2.7). Here, $\log_{10}$(repertoire) was positively correlated with relative HVC ($r^2 = 0.46$; $p = 1.3 \times 10^{-7}$) and MMAN ($r^2 = 0.26$; $p = 2.2 \times 10^{-4}$) volumes but not with other nuclei (all $p > 0.08$). Numerous multivariate models were then compared (Table 2.8). A full model that included all song nuclei performed significantly better than either of the previous correlations ($R^2 = 0.72$; ln ML = -17.7; LRT, both $p < 2.9 \times 10^{-5}$), and only HVC retained a significant coefficient (3.0; $p = 1.4 \times 10^{-7}$). Reduced models that contained nuclei along the caudal motor pathway were comparable to the full model, including those with all three nuclei (HVC, RA, nXIIts; $R^2 = 0.71$; LRT, $p = 0.80$), HVC and RA ($R^2 = 0.67$; $p = 0.19$), and HVC and nXIIts ($R^2 = 0.67$; $p = 0.20$).

Table 2.6. Correlations between the volumes, neuron numbers, and neuron densities of HVC, RA, and nXIIts. The first three are bivariate correlations between the size factor and neuronal densities of the three nuclei. For each, df = 56. The remaining regressions are two-factor models with size [log(Telen-SS)] as a confounding variable. The partial *r* and corresponding p-values refer to each trait after accounting for size. For these models, df = 55.

X		Y	Trait	
Size	**Trait**		***r***	**p**
log(Telen-SS)	--	log(HVC density)	**-0.815**	**7.20E-15**
		log(RA density)	**-0.671**	**8.08E-09**
		log(nXIIts density)	**-0.708**	**5.14E-10**
log(Telen-SS)	log(HVC volume)	log(HVC neuron #)	**0.983**	**2.84E-42**
		log(HVC density)	-0.070	0.604
log(Telen-SS)	log(RA volume)	log(RA neuron #)	**0.915**	**2.54E-23**
		log(RA density)	**-0.465**	**2.71E-04**
log(Telen-SS)	log(nXIIts volume)	log(nXIIts neuron #)	**0.882**	**1.32E-19**
		log(nXIIts density)	**-0.559**	**6.06E-06**
log(Telen-SS)	log(HVC neuron #)	log(RA neuron #)	**0.583**	**1.96E-06**
		log(RA density)	**-0.464**	**2.75E-04**
log(Telen-SS)	log(RA neuron #)	log(nXIIts neuron #)	**0.524**	**2.91E-05**
		log(nXIIts density)	**-0.424**	**0.001**

Table 2.7. Two-factor models explaining the variation in log(repertoire). The volume of an individual structure and its corresponding size reference are explanatory variables, and the partial r, r^2, and p-values refer to each trait after accounting for size. The ln ML and AIC values describe the entire model. In all cases, df = 47.

X		Y	Trait			ln ML	AIC
Size	Trait		*r*	r^2	p		
log(Telen-SS)	**log(HVC)**	**log(syl rep)**	**0.677**	**0.458**	**1.30E-07**	**-33.066**	**74.131**
log(Telen-SS)	log(RA)	log(syl rep)	0.116	0.013	0.434	-47.728	103.457
log(Telen-SS)	log(Area X)	log(syl rep)	0.243	0.059	0.096	-46.565	101.130
log(Telen-SS)	log(LMAN)	log(syl rep)	0.040	0.002	0.788	-48.019	104.038
log(Telen-SS)	**log(MMAN)**	**log(syl rep)**	**0.510**	**0.260**	**2.15E-04**	**-40.687**	**89.375**
log(Telen-SS)	log(Uva)	log(syl rep)	0.150	0.022	0.310	-47.502	103.003
log(Telen-SS)	log(DM)	log(syl rep)	0.061	0.004	0.681	-47.967	103.933
log(Telen-SS)	log(nXIIts)	log(syl rep)	-0.251	0.063	0.085	-46.465	100.929
log(Body Mass)	log(Telen)	log(syl rep)	0.183	0.033	0.214		
log(T-MP)	log(MP)	log(syl rep)	-0.080	0.006	0.591		
log(T-LimbS)	log(Hp)	log(syl rep)	-0.113	0.013	0.446		
log(T-LimbS)	log(Sep)	log(syl rep)	-0.058	0.003	0.697		
log(T-LimbS)	log(TnA)	log(syl rep)	-0.117	0.014	0.429		
log(Telen)	log(MLd)	log(syl rep)	0.021	4.60E-04	0.885		

Table 2.8. Parameters of some multivariate GLS models with log(repertoire) as the response variable. Each column represents a separate model with various combinations of log-transformed nucleus volumes as explanatory variables and log(Telen-SS) as a confounding variable. Likelihood ratio tests are in comparison to the full model (left column).

		log(rep)	log(rep)	log(rep)	log(rep)	log(rep)
	coef.	0.619	0.553	-0.277	0.400	-0.305
log(Telen-SS)	*F*	0.622	1.575	0.411	0.756	0.458
	p	0.435	0.216	0.525	0.389	0.502
	coef.	**2.999**	**2.680**	**2.803**	**2.904**	**2.806**
log(HVC)	*F*	**41.087**	**72.395**	**60.063**	**83.403**	**55.227**
	p	**1.40E-07**	**7.60E-11**	**9.24E-10**	**8.24E-12**	**2.35E-09**
	coef.	-1.129	**-1.440**	--	**-2.393**	--
log(RA)	*F*	2.262	**5.898**	--	**25.576**	--
	p	0.141	**0.019**	--	**7.60E-06**	--
	coef.	-0.551	--	-0.910	--	**-1.717**
log(Area X)	*F*	0.887	--	2.429	--	**12.768**
	p	0.352	--	0.126	--	**0.001**
	coef.	0.016	--	**-1.000**	--	--
log(LMAN)	*F*	0.001	--	**5.051**	--	--
	p	0.975	--	**0.030**	--	--
	coef.	-0.178	--	--	--	--
log(MMAN)	*F*	0.148	--	--	--	--
	p	0.702	--	--	--	--
	coef.	-0.358	--	--	--	--
log(Uva)	*F*	0.134	--	--	--	--
	p	0.716	--	--	--	--
	coef.	0.337	--	--	--	--
log(DM)	*F*	0.165	--	--	--	--
	p	0.687	--	--	--	--
	coef.	**-1.162**	**-1.159**	--	--	--
log(nXIIts)	*F*	**4.396**	**6.038**	--	--	--
	p	**0.043**	**0.018**	--	--	--
	coef.	-1.852	-1.784	1.627	-0.299	2.894
intercept	*F*	0.300	1.766	1.947	0.056	7.376
	p	0.587	0.191	0.170	0.814	0.009
	df	39	44	44	45	45
	R^2	0.723	0.710	0.638	0.670	0.597
	ln ML	-17.708	-18.889	-24.284	-22.040	-26.946
	AIC	57.415	49.779	60.567	54.080	63.892
	LRT p	--	**0.797**	0.022	**0.193**	0.005

Table 2.8 (Continued).

		log(rep)	log(rep)	log(rep)	log(rep)	log(rep)
	coef.	-0.483	**-0.969**	-0.421	-0.694	0.182
log(Telen-SS)	*F*	1.332	**4.153**	0.365	0.843	0.174
	p	0.255	**0.047**	0.549	0.363	0.679
	coef.	**2.483**	**1.826**	**1.964**	**1.862**	**2.169**
log(HVC)	*F*	**67.361**	**16.424**	**38.070**	**38.052**	**77.290**
	p	**1.72E-10**	**1.98E-04**	**1.75E-07**	**1.75E-07**	**2.50E-11**
	coef.	--	--	--	--	--
log(RA)	*F*	--	--	--	--	--
	p	--	--	--	--	--
	coef.	--	--	--	--	--
log(Area X)	*F*	--	--	--	--	--
	p	--	--	--	--	--
	coef.	**-1.426**	--	--	--	--
log(LMAN)	*F*	**16.031**	--	--	--	--
	p	**2.30E-04**	--	--	--	--
	coef.	--	0.020	--	--	--
log(MMAN)	*F*	--	0.002	--	--	--
	p	--	0.967	--	--	--
	coef.	--	--	-0.965	--	--
log(Uva)	*F*	--	--	1.083	--	--
	p	--	--	0.304	--	--
	coef.	--	--	--	-0.454	--
log(DM)	*F*	--	--	--	0.207	--
	p	--	--	--	0.651	--
	coef.	--	--	--	--	**-1.907**
log(nXIIts)	*F*	--	--	--	--	**-5.077**
	p	--	--	--	--	**7.12E-06**
	coef.	1.491	4.150	1.698	2.949	-1.220
intercept	*F*	1.593	9.940	0.430	1.095	0.768
	p	0.213	0.003	0.515	0.301	0.386
	df	45	45	45	45	45
	R^2	0.597	0.618	0.482	0.494	0.485
	ln ML	-26.946	-25.600	-33.065	-32.483	-32.953
	AIC	63.892	61.200	76.129	74.966	75.906
	LRT p	0.015	2.87E-05	4.79E-05	3.17E-05	**0.202**

Table 2.8 (Continued).

		log(rep)	log(rep)	log(rep)	log(rep)	log(rep)
log(Telen-SS)	coef.	-0.127	0.609	0.009	-0.303	0.768
	F	0.036	0.738	0.000	0.259	1.373
	p	0.851	0.395	0.989	0.614	0.248
log(HVC)	coef.	--	--	--	--	--
	F	--	--	--	--	--
	p	--	--	--	--	--
log(RA)	coef.	-0.522	**1.933**	--	--	--
	F	0.839	**7.425**	--	--	--
	p	0.365	**0.009**	--	--	--
log(Area X)	coef.	--	--	**1.656**	-0.433	**1.609**
	F	--	--	**5.131**	0.586	**9.835**
	p	--	--	**0.028**	0.448	**0.003**
log(LMAN)	coef.	--	--	-1.013	--	--
	F	--	--	2.243	--	--
	p	--	--	0.141	--	--
log(MMAN)	coef.	**1.660**	--	--	**1.726**	--
	F	**16.100**	--	--	**12.946**	--
	p	**2.24E-04**	--	--	**0.001**	--
log(Uva)	coef.	--	--	--	--	--
	F	--	--	--	--	--
	p	--	--	--	--	--
log(DM)	coef.	--	--	--	--	--
	F	--	--	--	--	--
	p	--	--	--	--	--
log(nXIIts)	coef.	--	**-2.318**	--	--	**-1.947**
	F	--	**10.200**	--	--	**10.060**
	p	--	**0.003**	--	--	**0.003**
intercept	coef.	3.136	-2.173	0.102	3.964	-3.285
	F	2.733	1.015	0.003	6.529	2.594
	p	0.105	0.319	0.954	0.014	0.114
	df	45	45	45	45	45
	R^2	0.306	0.232	0.144	0.302	0.266
	ln ML	-40.235	-42.723	-45.373	-40.370	-41.622
	AIC	90.470	95.446	100.747	90.741	93.244
	LRT p	4.56E-08	4.64E-09	3.97E-10	4.03E-08	1.28E-08

Table 2.8 (Continued).

		log(rep)	log(rep)	log(rep)	log(rep)	log(rep)
log(Telen-SS)	coef.	-0.202	**1.383**	-1.113	-0.467	0.420
	F	0.132	**4.453**	1.691	0.276	0.485
	p	0.719	**0.040**	0.200	0.602	0.490
log(HVC)	coef.	--	--	--	--	--
	F	--	--	--	--	--
	p	--	--	--	--	--
log(RA)	coef.	--	--	--	--	--
	F	--	--	--	--	--
	p	--	--	--	--	--
log(Area X)	coef.	--	--	--	--	--
	F	--	--	--	--	--
	p	--	--	--	--	--
log(LMAN)	coef.	-0.704	0.986	--	--	--
	F	2.535	3.049	--	--	--
	p	0.118	0.088	--	--	--
log(MMAN)	coef.	**1.774**	--	**1.473**	**1.499**	**1.631**
	F	**19.116**	--	**15.524**	**15.572**	**21.469**
	p	**7.19E-05**	--	**2.81E-04**	**2.76E-04**	**3.08E-05**
log(Uva)	coef.	--	--	0.918	--	--
	F	--	--	0.829	--	--
	p	--	--	0.367	--	--
log(DM)	coef.	--	--	--	-0.062	--
	F	--	--	--	0.003	--
	p	--	--	--	0.958	--
log(nXIIts)	coef.	--	**-1.818**	--	--	**-1.360**
	F	--	**6.196**	--	--	**7.506**
	p	--	**0.017**	--	--	**0.009**
intercept	coef.	2.938	-3.327	6.698	4.012	0.196
	F	3.040	2.316	4.474	1.423	0.009
	p	0.088	0.135	0.040	0.239	0.924
	df	45	45	45	45	45
	R^2	0.331	0.162	0.306	0.293	0.394
	ln ML	-39.345	-44.859	-40.240	-40.686	-36.908
	AIC	88.690	99.717	90.481	91.372	83.816
	LRT p	1.03E-07	6.41E-10	4.54E-08	3.02E-08	9.38E-07

A surprising pattern among partial coefficients emerged in the reduced models: several nuclei whose relative volumes alone were not related to repertoire (RA, Area X, LMAN, nXIIts) became significant explanatory variables after the variation associated with other area(s) had been factored out. Moreover, the sign of these partial slopes differed such that the most afferent nucleus relative to the syrinx was positive while those of efferent area(s) were usually negative. This pattern was evident in multiple combinations of nuclei and robust to the use of different trees. The only exception was HVC, which always had a positive coefficient, even when paired with MMAN or Uva (both $p < 2.0 \times 10^{-4}$). MMAN had a positive partial slope in every case except when paired with HVC, in which case it was not significant ($p = 0.97$, all other $p < 0.001$). Conversely, nXIIts had a negative coefficient when paired with every telencephalic nucleus (all $p < 0.02$) and nearly significant negative slopes with Uva and DM (both $p < 0.08$). The sign of coefficients for other nuclei depended on the nucleus with which it was paired; RA (-2.4), Area X (-1.7), and LMAN (-1.4; all $p < 0.001$) all had negative partial slopes when paired with HVC, but RA had a positive slope when paired with nXIIts (1.9, $p = 0.009$), as did Area X when paired with LMAN (1.7, $p = 0.03$) or nXIIts (1.6, $p = 0.003$). The same pattern was present in models that used HVC, RA, and nXIIts neuron numbers as explanatory variables (Table 2.9). Here, all models performed slightly less well than their volumetric counterparts, though the full model nevertheless explained a large portion of the observed behavioral variation ($R^2 = 0.64$; AIC = 59.9).

These opposing relationships suggested that the degree of convergence along the motor pathway rather than its overall relative volume is more closely associated with syllable repertoire size. This was assessed with residual analyses whereby the size of one nucleus relative to another or to the telencephalon was first calculated and then regressed with repertoire size (Table 2.10). The volume of HVC relative to RA

Table 2.9. Parameters of some multivariate GLS models with log(repertoire) as the response variable. Each column represents a separate model with a size reference and various combinations of log-transformed nucleus neuron numbers as explanatory variables. Likelihood ratio tests are in comparison to the full model (left column).

		log(rep)	**log(rep)**	**log(rep)**	**log(rep)**	**log(rep)**	**log(rep)**	**log(rep)**
log(Telen-SS)	coef.	0.412	0.349	0.054	0.719	-0.510	0.646	**1.189**
	F	0.943	0.608	0.019	1.221	1.438	0.915	**4.603**
	p	0.337	0.440	0.892	0.275	0.237	0.344	**0.037**
log(HVC n#)	coef.	**2.331**	**2.410**	**2.047**	--	**1.850**	--	--
	F	**62.417**	**60.671**	**58.866**	--	**37.836**	--	--
	p	**5.61E-10**	**7.00E-10**	**1.04E-09**	--	**1.72E-07**	--	--
log(RA n#)	coef.	-1.107	**-1.812**	--	1.010	--	0.146	--
	F	3.972	**13.043**	--	1.822	--	0.049	--
	p	0.052	**0.001**	--	0.184	--	0.826	--
log(nXIIts n#)	coef.	**-1.369**	--	**-1.940**	**-1.844**	--	--	-1.233
	F	**6.056**	--	**15.564**	**4.705**	--	--	2.884
	p	**0.018**	--	**2.77E-04**	**0.035**	--	--	0.096
intercept	coef.	-2.660	-4.169	-3.111	1.163	-6.582	-0.719	2.252
	F	3.452	9.339	4.544	0.315	24.277	0.135	1.369
	p	0.070	0.004	0.039	0.577	1.12E-05	0.715	0.248
	df	44	45	45	45	46	46	46
	R^2	0.643	0.594	0.611	0.137	0.476	0.046	0.102
	ln ML	-23.957	-27.117	-26.075	-45.595	-33.352	-48.032	-46.568
	AIC	59.915	65.629	62.150	101.190	74.704	104.063	101.135
	LRT p	0.012	0.040	4.75E-11	8.31E-05	3.51E-11	1.52E-10	0.012

Table 2.10. Parameters of some multivariate GLS models with log(repertoire) as the response variable. Each column represents a separate model with various combinations of relative nucleus volumes as explanatory variables. Volumes are relative to another nucleus (e.g., HVC_RA is HVC relative to RA) or to Telen-SS.

		log(rep)	log(rep)	log(rep)	log(rep)	log(rep)	log(rep)
HVC_RA	coef.	**2.632**	**2.891**	--	--	--	--
	F	**71.522**	**72.499**	--	--	--	--
	p	**6.33E-11**	**4.39E-11**	--	--	--	--
RA_nXIIts	coef.	**1.442**	--	**2.218**	--	--	--
	F	**13.037**	--	**13.030**	--	--	--
	p	**7.52E-04**	--	**7.42E-04**	--	--	--
HVC_T-SS	coef.	--	--	--	**1.879**	--	--
	F	--	--	--	**40.044**	--	--
	p	--	--	--	**8.57E-08**	--	--
RA_T-SS	coef.	--	--	--	--	0.592	--
	F	--	--	--	--	1.004	--
	p	--	--	--	--	0.322	--
nXIIts_T-SS	coef.	--	--	--	--	--	-0.925
	F	--	--	--	--	--	2.383
	p	--	--	--	--	--	0.129
intercept	coef.	-0.592	0.302	-0.216	5.384	2.857	-1.491
	F	3.169	1.473	0.172	59.954	3.207	0.658
	p	0.082	0.231	0.680	6.18E-10	0.080	0.421
	df	46	47	47	47	47	47
	R^2	0.694	0.607	0.217	0.460	0.021	0.048
	ln ML	-20.221	-26.334	-43.201	-34.098	-48.678	-47.985
	AIC	48.441	59.201	92.403	74.195	103.357	101.969

was strongly and positively related to repertoire (r^2 = 0.61; AIC = 59.2; p = 4.4×10^{-11}) and was a significantly better predictor than HVC relative to telencephalon (r^2 = 0.46; AIC = 74.2; Figure 2.3). The size of RA relative to nXIIts was also positively correlated with repertoire (r^2 = 0.22; AIC = 92.4; p = 7.4×10^{-4}) despite the fact that neither RA nor nXIIts volumes relative to telencephalon were related to it (both p > 0.13). Interestingly, the HVC-to-RA and RA-to-nXIIts values were not correlated (r = 0.23, p = 0.11) and both retained significant and positive partial coefficients in a two-factor model explaining the variation in repertoire (HVC-to-RA: 2.6, p = 6.3×10^{-11}; RA-to-nXIIts: 1.4, p = 7.5×10^{-4}). This model was one of the best as judged by the AIC (R^2 = 0.69; AIC = 48.4); it was comparable to that containing HVC, RA, and nXIIts with T-SS as a confounding size variable (R^2 = 0.71; AIC = 49.8).

(d) *Song and other brain structures*

Syllable repertoire size was not correlated with the volumes of the telencephalon (r = 0.18, p = 0.21), MP (r = -0.08, p = 0.59), Hp (r = -0.11, p = 0.45), Sep (r = -0.06, p = 0.70), TnA (r = -0.12, p = 0.43), or MLd (r = 0.02, p = 0.88) after accounting for overall size (Table 2.7).

Discussion

Behavioral specializations are often associated with hypertrophy of their underlying neural substrate. Examples are widespread and include brain structures related to auditory specializations (Kubke et al. 2004; Iwaniuk et al. 2006), echo- and electrolocation (Clark et al. 2001), binocular vision (Barton 2004; Iwaniuk et al. 2008), visual motor coordination (Iwaniuk & Wylie 2007), and even more abstract capabilities such as spatial memory (Krebs et al. 1989), feeding innovations (Timmermans et al. 2000), and complex social interactions (Whiten & Byrne 1988; Dunbar 1995; Burish et al. 2004). The nature of these brain-behavior relationships is complex and many of their intricacies remain unclear, but the enhanced behavioral

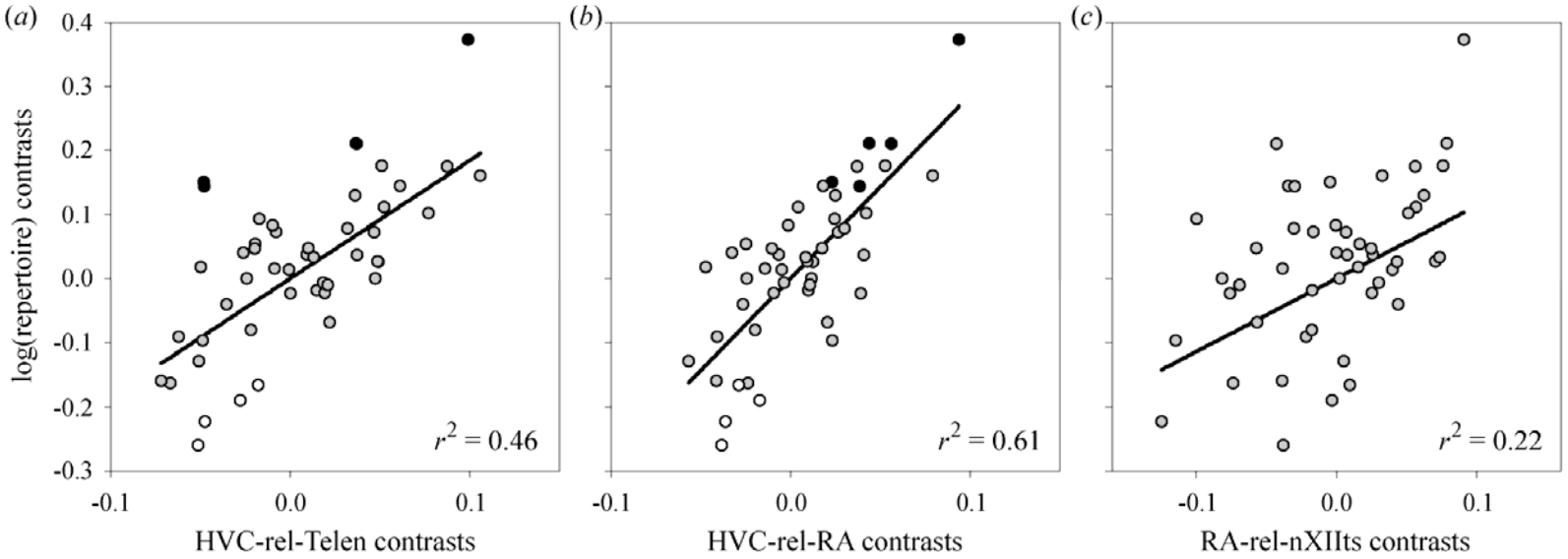


Figure 2.3. Correlations between standardized independent contrasts of $\log_{10}$(syllable repertoire) and (*a*) HVC volume relative to the telencephalon, (*b*) HVC volume relative to RA, and (*c*) RA volume relative to nXIIts. Black and open symbols in (*a*) to (*b*) highlight the reduction of the largest positive and negative deviations, respectively, when repertoire is correlated with HVC relative to RA rather than the telencephalon.

capacities seem likely to result from a larger region's increased processing power and/or its expanded influence over other areas (Striedter 2005). These possibilities are difficult to dissociate because they are not mutually exclusive, are almost certainly linked, and their relative roles are likely to vary with the behavior produced. Moreover, the entire pathways relevant to a particular behavior are often unknown or their complete measurement is impractical. As a result, the evolutionary relationships between behaviors of interest and the structure of their underlying neural circuits have gone largely unexplored.

The neurobiology of birdsong provides distinct advantages in this regard. First, song is a conspicuous and quantifiable behavior that circumvents the need for categorical classifications of 'specialists' or 'generalists'. Second, the song system is well characterized and the functional roles of several areas are known in detail. The premotor nucleus HVC encodes song sequence (Hahnloser et al. 2002), and its volume is positively related to the repertoire size both within and across species (DeVoogd et al. 1993; Garamszegi & Eens 2004). The present study replicates these findings across a wide phylogeny of mostly unstudied songbirds. However, some species deviate substantially from this regression line and relative HVC size cannot be said to accurately predict their capacity (or lack thereof) for complex songs. Inclusion of downstream nuclei in the present analyses accounts for these discrepancies remarkably well, and it does so in a surprising way because these relative volumes are inversely correlated with the residuals from the HVC-repertoire regression. Thus, species that have fewer HVC neurons than expected from their repertoire also have few RA and nXIIts neurons, while those with many more HVC neurons than expected from their repertoire have correspondingly high neuron numbers in RA and nXIIts. This indicates that repertoire size may not be regulated by the size of its underlying neural

circuit *per se* and instead highlights the potential importance of top-down control in the encoding of this complex motor behavior.

Syllables are an operationally convenient unit for behavioral quantification, but they can also obscure details about the nature of the observed vocal diversity. For example, one strategy for building a larger repertoire might be to produce a more diverse array of acoustically distinct sounds, while another might be to modify the temporal properties or sequence of sounds already mastered. Both would effectively produce new, distinct syllables, but they seem likely to involve different physiological mechanisms. Evolutionary changes in convergence from HVC to RA and RA to nXIIts are independent and may represent two means by which these behavioral differences arise. In the zebra finch, 40% of all HVC neurons project to RA and this proportion is remarkably stable across individuals (Ward et al. 2001). These cells sparsely encode song whereby each neuron produces a short (~6 ms), temporally precise burst of action potentials that corresponds to one specific syllable segment, and the sequential activity of these neurons ultimately specifies motor output (Hahnloser et al. 2002; Long & Fee 2008; Prather et al. 2008). This correspondence between single neurons and brief sounds highlights a mechanism by which increases in HVC neuron number could enhance syntactical capacities, even if many share similar axonal projections onto a given number of RA neurons. More HVC neurons will create more activity chain possibilities, which will increase the number of ways that short sounds can be combined. Conversely, decreases in the number of HVC neurons that can excite a particular RA neuron will decrease the number of ways that neuron can be incorporated into a particular song sequence.

Individual RA neurons burst multiple times throughout each song rendition, but population-level activity patterns are specific to short syllable segments and are equally temporally precise as activity in HVC (Yu & Margoliash 1996; Leonardo &

Fee 2005). RA also presents the first myotopic map of syringeal and respiratory muscles along this pathway (Vicario 1991; Wild 1993), and syringeal labial adduction or abduction and the airflow past them largely shape song spectral features (Goller & Suthers 1996). It was recently suggested that the strength of each muscular contraction is determined by the linear sum of convergent RA inputs onto nXIIts neurons rather than the specific identity of active RA ensembles (Fee et al. 2004; Leonardo & Fee 2005). If true, evolutionary increases in RA-to-nXIIts convergence could expand the range of attainable nXIIts firing rates (and thus the contractile strength of each motor unit) and/or enhance contractile precision if accompanied by decreased synaptic weights and/or axonal arborization. Such changes would increase the level of control over syringeal shape and could permit the production of more spectrally varied sounds. These ideas are admittedly speculative because many interspecific differences in circuit structure could affect the proposed relationships, including the number of syringeal muscle fibers, motor unit size, synaptic weights, axonal branching, convergence onto single neurons, and physiological activity patterns. Nevertheless, evolutionary changes in repertoire size are closely related to the degree of convergence along the caudal motor pathway, and current hypotheses on song encoding provide clues as to why this might be the case.

Much less is known about the functions of areas afferent to HVC and, incidentally, the mechanisms underlying song organization and song type switching. In the zebra finch, sequential activity of HVC neurons appears to be determined by intrinsic connectivity within the nucleus itself (Long & Fee 2008). Yet, most other species show far more behavioral variability and many can organize syllables into distinct song types or produce continuous strings of numerous syllables (e.g., Beecher et al. 2000; Briefer et al. 2008). It is not known how these feats are accomplished, but they seem likely to involve areas afferent to HVC given its role in syllable encoding.

The thalamic nucleus Uva supplies feedback to HVC that appears critical for inter-hemispheric coordination (Coleman & Vu 2005). The relative volume of Uva is positively correlated with that of HVC across species, but its deviations from allometric expectations are quite small. Insofar as the extensive interspecific variability in song organization can be expected to accompany large anatomical differences, Uva appears unlikely to serve such a role. The telencephalic nucleus MMAN also projects to HVC but its function is less clear. In the zebra finch, MMAN axons ramify throughout HVC and lesions in juveniles both severely disrupt song learning and prevent song stabilization (Foster et al. 1997; 2001). The effects of lesions made in adults are much more subtle, but they do increase syntactical variability and particularly affect the first syllable in a song. In light of its strong correlation with HVC and considerable variability across species, MMAN appears well positioned to function in syllable and/or song type organization.

We also tested for patterns of correlated evolution between repertoires and other neural traits. In the zebra finch, repertoire size is correlated with HVC volume, which is heritable and related to overall telencephalon size (Airey et al. 2000; Airey & DeVoogd 2000). Across species, however, repertoires are not related to relative telencephalon size (Garamszegi & Møller 2004) or to the relative volumes of structures involved in other behaviors, including MP, a telencephalic subdivision correlated with feeding innovation rate (Timmermans et al. 2000); Hp, which is involved in spatial memory (Krebs et al. 1989); Sep, which is involved in sociality and territoriality (Goodson et al. 1999; 2006); TnA, which underlies aspects of sexual behavior and pair bonding (Thompson et al. 1998; Svec et al. 2009); or MLd, which encodes general sound properties (Woolley & Casseday 2005). Thus, elaborate syllable repertoires do not appear to have evolved in coordination with brain areas underlying other behaviours and cognitive tasks.

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CHAPTER 3

EVOLUTION OF FUNCTIONAL NEURAL CIRCUITS IN THE OSCINE BRAIN

Abstract

Two models of brain evolution have been proposed to explain the source of changes in brain composition. The model of developmental conservation asserts that neurogenetic schedules are conserved between species but are altered in duration. As a result, late-developing areas become disproportionately large as overall brain size increases because the number of neuronal precursors increases exponentially. The model of mosaic evolution focuses on coordinated changes between regions, and specifically predicts that functionally connected areas change in concert but independently of other areas. The present study tests predictions of the two models at the level of functional neural circuits across a wide phylogeny of songbirds. Interestingly, the data support elements of both. Telencephalic nuclei develop later in ontogeny and possess greater allometric slopes than nuclei in the thalamus and brainstem. Functionally connected nuclei evolve in concert as a result of coordinated changes in relative neuron number. Finally, the song system is much more variable than other systems, which may be related to its late development relative to surrounding tissues.

Introduction

The vertebrate brain comes in many shapes and sizes, and little is currently known about the evolutionary processes that give rise to these various forms. In particular, considerable debate has centered on differences in brain composition between large and small animals and how differences may relate to ecological adaptations and/or phylogenetic relationships. This research has focused on two main arguments. The 'developmental conservation' hypothesis asserts that brain structure is

largely determined by its sequence of development, which supports graceful scaling of brain components with brain and body size (Finlay & Darlington 1995; Finlay et al. 2001). According to this view, neurogenesis schedules for different brain regions are conserved across species but altered in duration, therefore compositional differences between large and small brains are predictable from their developmental program (Clancy et al. 2001). Behavioral evolution is therefore thought to occur within the context of these conserved developmental mechanisms, which promote computationally efficient structural changes by means of graceful scaling and the allocation of new functions to favorable locations. By contrast, a 'mosaic' model argues that selective forces drive specific changes to individual systems and/or brain regions and that these forces are strong compared to those that might favor conserved developmental programs (Barton & Harvey 2000; de Winter & Oxnard 2001). This hypothesis contends that grade shifts, relatively large and abrupt changes in brain composition, occur regularly and can explain the majority of evolutionary changes in brain structure. While competing, these hypotheses are not exclusive. They instead differ in their assertions about the degree to which brain evolution is concerted or piecemeal and about the frequency with which large grade shifts occur (Striedter 2005).

Nearly this entire debate stems from measures of gross brain subdivisions in mammals. The case for concerted brain evolution is based on attempts to explain the covariation among these different components in terms of the total observed variation (Finlay & Darlington 1995; Reep et al. 2007). Not surprisingly, the vast majority of variation is explained by overall brain size. The proportional sizes of different regions are not constant across brains, however, and these differences are closely predicted by neurogenetic schedules. Areas that continue to develop late in ontogeny (e.g., telencephalon) possess larger allometric slopes and thus constitute a greater proportion

of larger brains. The olfactory bulb and limbic structures (e.g., subicular cortices, hippocampus, septum) deviate from this general rule but covary with each other, an effect that could arise from a common developmental source such as the expansion or contraction of prosomeric boundaries (Puelles & Rubenstein 2003; Reep et al. 2007). The remaining variance left unexplained by these two factors (~1%) might be associated with behavioral specializations in individual species, which permits up to ~2.5-fold differences in species of similar size. Thus, the model of concerted brain evolution seeks to explain the total variation in brain composition and identifies developmental events, such as the timing of neurogenesis and/or the size of neuronal precursor pools, as its primary determinants. It further contends that graceful scaling of brain regions is a generally more efficient means to effect behavioral change than the independent restructuring of individual brain components.

The second approach has focused on differential changes in specific brain areas or brain composition and their relationships to behavioral or ecological specializations (e.g., de Winter & Oxnard 2001). The literature is filled with examples of brain-behavior relationships that span sensory (Welker et al. 1964; Kubke et al. 2004), motor (DeVoogd et al. 1993; Yopak et al. 2007), and even fairly abstract cognitive capacities (Krebs et al. 1989; Emery & Clayton 2004; Lefebvre et al. 2004). Moreover, evolutionary changes in brain structure reflect its connectivity, with interconnected regions changing in parallel but seemingly independently of other areas (Barton & Harvey 2000; Whiting & Barton 2003). Of central importance, then, is the magnitude of these behavior-related differences and the frequency with which large-scale changes occur. Clear examples of mosaic changes exist, including large isocortical volume differences between primates and insectivores (Stephan et al. 1981) and the nearly 10-fold difference in superior colliculus volumes between the ground squirrel and rat (Woolsey et al. 1971). Such large differences have usually been

limited to distantly related species, however, as brain composition appears quite stable within closely related taxa (Clark et al. 2001). Analyses in other groups of animals can shed light on the frequency with which these large changes occur and the applicability of either model across vertebrates.

Recent studies on the avian brain have highlighted several instances of mosaic evolution. Brain composition varies widely between species and often reflects both phylogeny and behavioral and/or ecological specializations (Boire & Baron 1994; Burish et al. 2004; Iwaniuk & Hurd 2005). These large differences may arise through any of several changes to developmental programs, including delayed and/or prolonged periods of neurogenesis, increased progenitor cell cycle rates, and expanded proliferative cell regions (Charvet & Striedter 2008, 2009a; Striedter & Charvet 2008). Interestingly, the strategies employed reflect developmental mode, with altricial but not precocial species extending brain development (Charvet & Striedter 2009b). Thus, large grade shifts are evident between avian orders and multiple differences in developmental programs appear to underlie these changes. Evolutionary changes within orders have not been characterized, however, therefore it remains unknown if gradual scaling exists in birds as in mammals.

To the best of our knowledge, no study has actually measured the evolutionary patterns of discrete functional circuits with respect to these questions. The present study does so in songbirds, whose nuclear (rather than laminar) pallial organization facilitates the measurement of telencephalic areas with known connectivity and function. It also provides an opportunity to ascertain the cellular basis of correlations between connected brain regions (Barton & Harvey 2000; Whiting & Barton 2003; Iwaniuk et al. 2004). These relationships could result from any of three causes, including (1) changes in cell density of one region due to more or fewer axons from afferent sources, (2) trophic-induced cell growth resulting from increased afferent

activity, or (3) coordinated changes in neuron number (Finlay et al. 2001). While the third is commonly assumed to be the primary cause, this has not been explicitly shown. Finally, songbirds are capable of many diverse and complex behaviors that include learned songs (Marler & Slabbekoorn 2004), intricate social interactions (Burish et al. 2004), food caching and tool use (Emery & Clayton 2004), and various innovations to cope with variable seasonal and environmental demands (Lefebvre et al. 2004). This study examines whether functional pathways in the songbird brain exhibit similar amounts of diversity.

Methods

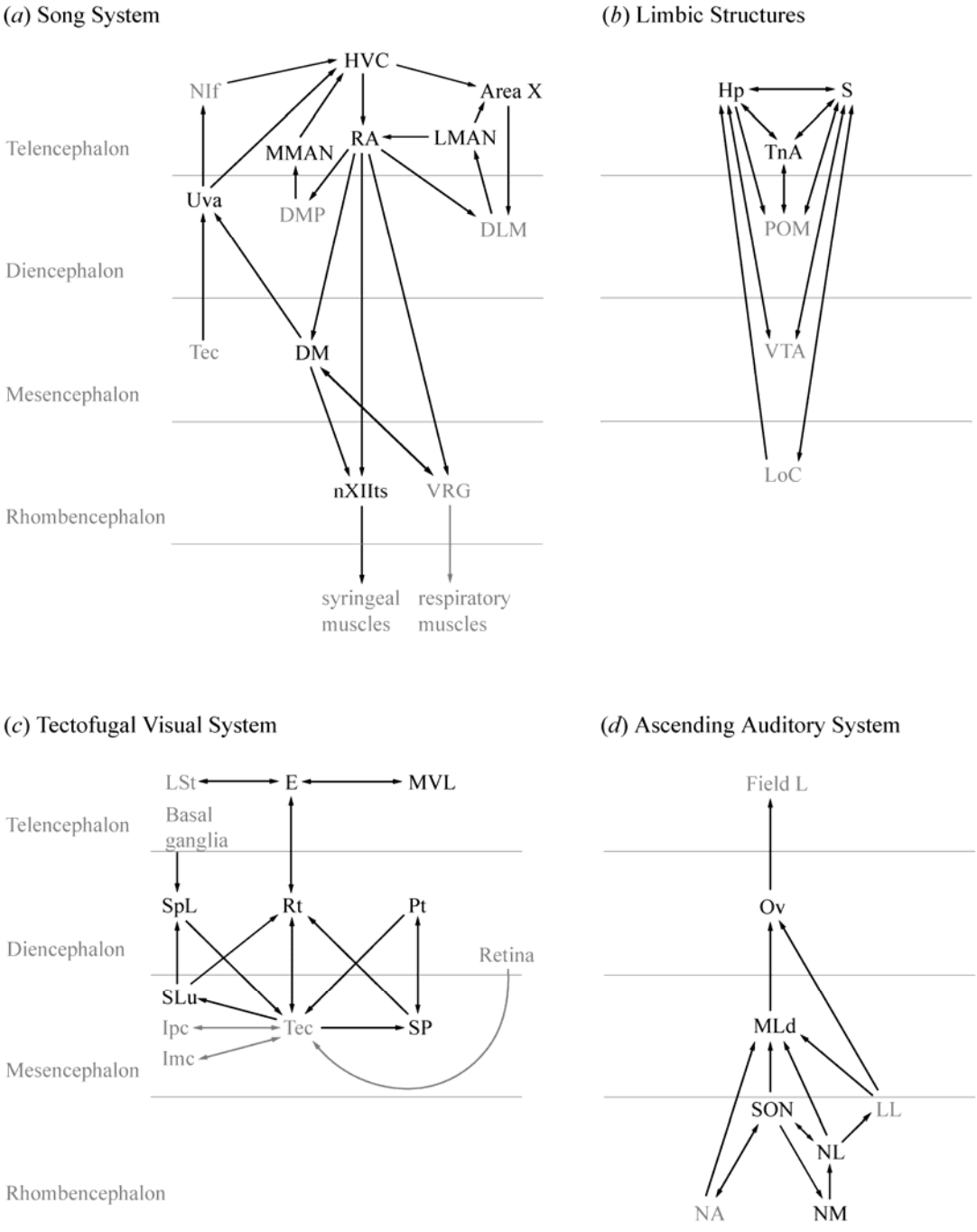
(a) *Specimen collection and preparation*

A portion of the data presented here were the subject of a previous study (Moore et al. 2009), and new measurements were obtained from the same brains. Specimens include 58 temperate zone species that were wild-caught under permit with mist nets during spring months (April – June) when in reproductive condition. At the time of capture, birds were deeply anesthetized with a barbiturate and transcardially perfused with 0.8% saline followed by 10% formalin in saline. Brains were extracted, post-fixed for at least 24 hours, cryoprotected with 30% sucrose/10% formalin, embedded in gelatin, and sectioned at 40 μm in the coronal plane with a freezing, sliding microtome. Sections were then mounted onto gel-coated slides and Nissl-stained with cresyl violet. All procedures were approved by the Cornell University Institutional Animal Care and Use Committee.

(b) *Brain measurements*

In total, 23 discrete nuclei from 4 functional systems (song, limbic, tectofugal visual, ascending auditory) and spanning 4 major brain subdivisions (telencephalon, diencephalon, midbrain, hindbrain) were measured (Figure 3.1). Song nuclei included HVC (abbreviation used as proper name) in the nidopallium, robust nucleus of the

Figure 3.1. Diagram of major connections between nuclei in the (*a*) song system, (*b*), limbic system, (*c*) tectofugal visual system, and (*d*) ascending auditory system. Nuclei that were measured are shown in black while those that were not are in gray.


(a) Song System
HVC
NIf
Area X
Telencephalon
RA
LMAN
MMAN
Uva
DMP
DLM
Diencephalon
Tec
DM
Mesencephalon
nXIIts
VRG
Rhombencephalon
syringeal muscles
respiratory muscles
(b) Limbic Structures
Hp
S
TnA
POM
VTA
LoC
(c) Tectofugal Visual System
LSt
E
MVL
Telencephalon
Basal ganglia
SpL
Rt
Pt
Diencephalon
Retina
SLu
Ipc
Tec
SP
Imc
Mesencephalon
Rhombencephalon
(d) Ascending Auditory System
Field L
Ov
MLd
SON
LL
NL
NA
NM

arcopallium (RA), Area X of the striatum, the lateral and medial magnocellular nuclei of the nidopallium (LMAN and MMAN, respectively), uvaeformis of the thalamus (Uva), the dorsomedial nucleus of the intercollicular complex (DM) in the midbrain, and the tracheosyringeal portion of the hypoglossal nucleus (nXIIts) in the hindbrain. Major projections of the song system are well established and nuclear boundaries were unambiguous (Nottebohm et al. 1976; 1982; Moore et al. 2009). Nuclei shown in Figure 3.1 but not measured include the nucleus interface of the nidopallium (NIf), the dorsomedial nucleus of the posterior thalamus (DMP), the medial portion of the dorsolateral nucleus of the anterior thalamus (DLM), and nuclei within the ventral respiratory group (VRG).

Limbic structures included the hippocampus (Hp), septum (Sep), and nucleus taeniae of the amygdala (TnA), all of which are in the telencephalon and interconnected (Székely & Krebs 1996; Cheng et al. 1999; Atoji & Wild 2004). The lateral extent of Hp was identified by its lower cell density compared to the adjacent parahippocampal area. Caudal Hp does not have clear boundaries, therefore measurements were arbitrarily stopped at the section in which the cerebellum reached the dorsal-most extent of the telencephalon. Sep boundaries were identified by differences in staining intensity and largely coincided with those described by chemoarchitecture (Goodson et al. 2004); the estimates reported here include most of the four major subdivisions but exclude portions of the nucleus of the diagonal band ventral to the septopallio-mesencephalic tract. TnA was delineated from surrounding arcopallium on the basis of its high cell density. Nuclei shown in Figure 3.1 but not measured are the medial preoptic area (POM), ventral tegmental area (VTA), and locus coeruleus (LoC).

Tectofugal visual nuclei were MVL (abbreviation used as a proper name) of the mesopallium, entopallium (E; including the perientopallial belt, Ep) in the

nidopallium, nuclei rotundus (Rt), pretectalis (Pt), and spiriformis lateralis (SpL) of the thalamus, and subpretectalis (SP) and nucleus isthmi pars semilunaris (SLu) of the midbrain. Connectivity between these areas has been described in detail (Hellmann et al. 2001; Theiss et al. 2003; Krützfeldt & Wild 2004). MVL was clearly distinguishable from surrounding mesopallium based on its small somata and high cell density, and E boundaries were determined from the relatively high staining intensity of the Ep belt. Boundaries of thalamic and mesencephalic nuclei were unambiguous. Areas shown in Figure 3.1 but not measured include the lateral striatum (LSt), nucleus isthmi pars parvocellularis (Ipc), nucleus isthmi pars magnocellularis (Imc), and the tectum (Tec).

Finally, nuclei along the ascending auditory pathway included nucleus ovoidalis (Ov) of the thalamus, mesencephalicus lateralis, dorsalis (MLd) of the midbrain, and the superior olivary (SON), laminar (NL), and magnocellular (NM) nuclei of the hindbrain (Carr & Konishi 1990; Vates et al. 1996; Burger et al. 2005). Telencephalic auditory areas [e.g., Field L, caudomedial nidopallium (NCM) or caudal mesopallium (CM)] do not have clear boundaries and could not be measured. MLd was distinguished from the surrounding intercollicular complex on the basis of cell density and staining intensity, all other areas were unambiguous. Nuclei shown in Figure 3.1 but not measured were Field L of the nidopallium, the lateral lemniscus (LL), and nucleus angularis (NA).

Alternate sections were viewed under 40× or every fourth section under 20× (Hp and E) magnification and nuclei were traced using a camera lucida. Unmagnified digital images of every fourth section were used to measure the telencephalon and entire brain. All measurements were made in one side of the brain, typically the left except in cases where torn tissue or poor staining precluded their measurement, in which case the entire structure was measured in the right. Cross-sectional areas of

scanned traces and brain images were measured using NIH ImageJ software (Rasband 2007) and final volumes were computed by summing the areas and multiplying by the sampling interval (0.08 or 0.16 mm).

Neuron densities were estimated in 11 nuclei from three systems. Presumptive neurons were discriminated from glia on the basis of their soma size, uniformly stained cytoplasm, and a single, darkly stained nucleolus. Nucleolus counts were tallied within sampling windows that were evenly distributed throughout each plane of each structure, and a cell was only counted if its nucleolus was within the grid. For nuclei RA and Rt, sampling grid dimensions were 120 × 120 μm (400×); for all other nuclei (HVC, LMAN, MMAN, nXIIts, Hp, Sep, E, SpL, SP), grid dimensions were 80 × 80 μm (600×). On average, 12 (range: 7-26) samples were collected from each region, and overall neuron density estimates were calculated by averaging the neuron counts and dividing by the volume sampled. One specimen from each species was sampled for three song nuclei (HVC, RA, nXIIts) whereas one specimen from approximately half of the species was sampled for all others. Species were chosen to maximize differences in overall brain size and relative nucleus volumes.

(c) *Data analysis*

Phylogenetically-based statistical analyses were used in all cases. A fully resolved composite phylogeny was constructed from published molecular studies and was the same as that used previously (Moore et al. 2009). For analyses of $\log_{10}$-transformed data, the phylogenetic tree was scaled using the arbitrary branch lengths method of Grafen (1989) and the rho transform ($\rho = 0.3$) based on this tree's superior performance in diagnostic tests (Garland et al. 1992; Blomberg et al. 2003). Allometric slopes and confidence intervals were estimated using a generalized least squares (GLS) approach, which is equivalent to analyses of independent contrasts when performed with the same tree (Garland & Ives 2000). To measure the allometric

slope for the telencephalon, the independent variable was brain volume minus telencephalon; for each nucleus it was brain volume minus the sum of all nuclei.

A factor analysis was performed on standardized independent contrasts of $\log_{10}$-transformed data to ascribe portions of variance to common components. Because factor analysis centers variables at their mean and contrasts must be centered at 0, contrasts for each variable were entered twice and the second entry was multiplied by -1, thus giving each a mean = 0 (and $n = 2*57 = 114$, therefore no significance tests were performed). For the subsequent model construction, axes were rotated using an oblique Oblimin rotation.

Finally, correlations between nuclei were calculated in two ways. Relationships between two nuclei were measured by constructing two-factor GLS models that explained the variation in one nucleus as a function of the other with size (i.e. brain – nuclei) as a confounding variable. The same form of model was used for analyses of neuron numbers and densities, and trees were pruned and re-scaled prior to those for which not every species was sampled. Multivariate models were also constructed whereby the variation in the volume of one nucleus was explained as a function of all others within that functional circuit. Finally, phylogenetic signal was measured in both log-transformed and relative trait volumes. The latter were computed with the formula $\log_{10}[\text{trait}/(\text{size}^b)]$, where b was the allometric exponent, and 'trait' and 'size' were original data values of a nucleus and size reference, respectively.

Mesquite v2.5 (Maddison & Maddison 2008) was used to manage data and trees, and the PDAP:PDTree v1.14 module was used for all analyses of independent contrasts (Garland et al. 1999; Garland & Ives 2000). Phylogenetic signal was computed with the Matlab program PHYSIGLL.m (Blomberg et al. 2003) and phylogenetic generalized least squares (PGLS) models were constructed with

REGRESSIONv2.m (Lavin et al. 2008). For these analyses, trees were first converted to variance-covariance matrices with the PDDIST module of Phenotypic Diversity Analysis Programs (Garland & Ives 2000).

Results

(a) *Allometry*

All log-transformed and most relative nucleus volumes exhibited significant phylogenetic signal, the latter of which are listed in Table 3.1, indicating that the data were better described by the hierarchical than star phylogeny. The telencephalon displayed hyperallometry relative to the rest of the brain (slope = 1.08; SE = 0.06; Table 3.2). This was also evident at the level of individual nuclei, where those in the telencephalon had significantly greater slopes than nuclei within the thalamus or brainstem (Figure 3.2; *t*-test, $p < 0.001$).

(b) *System variability*

A factor analysis was performed on independent contrasts of $\log_{10}$-transformed nucleus volumes, and two factors explained 81% of the total variance (Table 3.3a). Most nuclei loaded highly on the first, which explained 70.7% of the total variance and primarily reflects changes in overall brain size (Table 3.3b). However, the majority of song nuclei loaded more strongly on a second factor that accounted for an additional 10.5% of the total variance. The two factors were correlated ($r = 0.59$), suggesting that a 'size' component was present in the 'song system' factor but that much of the variability associated with this component was not attributable to overall brain size. This disparity between the song system and the three others is also evident from correlations between system volumes, where the value for each was calculated by summing the raw values of their respective nuclei. Correlations between the song system and each system ($0.55 \leq r \leq 0.64$) were notably weaker than those between the other systems ($0.78 \leq r \leq 0.88$).

Table 3.1. Phylogenetic signal of relative trait volumes. Tree branch lengths were scaled using the method of Grafen (1989) and the rho transform ($\rho = 0.3$). In all cases, df = 56. For additional discussion of phylogenetic signal, see Blomberg et al. (2003) and Revell et al. (2008).

Trait	MSE_0	MSE_{star}	MSE	obs. MSE_0/MSE	exp. MSE_0/MSE	*K*	p	ln lklhd	ln $lklhd_{star}$
rel_Telen	0.0063	0.0061	0.0063	0.9911	1.5183	0.6528	**0.020**	65.0298	65.9427
rel_HVC	0.0831	0.0828	0.0603	1.3772	1.5183	0.9071	**<0.001**	-0.3677	-9.5339
rel_RA	0.0296	0.0294	0.0292	1.0168	1.5183	0.6697	**0.007**	20.7243	20.4429
rel_Area X	0.0351	0.0351	0.0334	1.0519	1.5183	0.6928	**0.002**	16.7779	15.3626
rel_LMAN	0.0373	0.0370	0.0381	0.9774	1.5183	0.6438	**0.015**	12.9325	13.8317
rel_MMAN	0.0726	0.0724	0.0469	1.5477	1.5183	1.0194	**<0.001**	6.9353	-5.6416
rel_Uva	0.0051	0.0051	0.0064	0.7984	1.5183	0.5259	0.389	64.5757	71.2047
rel_DM	0.0070	0.0069	0.0055	1.2745	1.5183	0.8395	**<0.001**	69.2888	62.3579
rel_nXIIts	0.0210	0.0209	0.0253	0.8308	1.5183	0.5472	0.280	24.8271	30.3284
rel_Hp	0.0084	0.0077	0.0066	1.2593	1.5183	0.8294	**<0.001**	63.6107	59.1881
rel_Sep	0.0052	0.0048	0.0044	1.1780	1.5183	0.7759	**0.003**	75.6655	72.8376
rel_TnA	0.0103	0.0101	0.0081	1.2699	1.5183	0.8364	**<0.001**	57.7920	51.3859
rel_MVL	0.0130	0.0130	0.0120	1.0872	1.5183	0.7161	**0.001**	46.5562	44.1330
rel_E	0.0095	0.0095	0.0076	1.2523	1.5183	0.8248	**<0.001**	59.8981	53.3788
rel_Rt	0.0099	0.0099	0.0079	1.2514	1.5183	0.8242	**<0.001**	58.5218	52.0188
rel_Pt	0.0149	0.0149	0.0108	1.3782	1.5183	0.9078	**<0.001**	49.5505	40.2648
rel_SpL	0.0110	0.0110	0.0116	0.9478	1.5183	0.6242	**0.020**	47.4413	49.0100
rel_SP	0.0126	0.0126	0.0109	1.1622	1.5183	0.7655	**<0.001**	49.3009	45.0030
rel_SLu	0.0082	0.0082	0.0085	0.9688	1.5183	0.6381	**0.019**	56.5504	57.6091

Table 3.1 (Continued).

Trait	MSE_0	MSE_{star}	MSE	obs. MSE_0/MSE	exp. MSE_0/MSE	*K*	p	ln lklhd	ln $lklhd_{star}$
rel_Ov	0.0055	0.0055	0.0064	0.8620	1.5183	0.5678	0.149	64.6996	69.2641
rel_MLd	0.0088	0.0088	0.0094	0.9372	1.5183	0.6173	**0.024**	53.5522	55.4625
rel_SON	0.0041	0.0039	0.0038	1.0841	1.5183	0.7140	**0.003**	79.6769	78.8400
rel_NL	0.0063	0.0060	0.0048	1.3201	1.5183	0.8695	**<0.001**	73.0195	66.5477
rel_NM	0.0045	0.0044	0.0050	0.8870	1.5183	0.5842	0.096	71.6187	75.4874

Table 3.2. Parameters and statistics of allometric equations from GLS regressions of the telencephalon and each nucleus. The size reference for the telencephalon was log(Brain – Telen) and for each nucleus was log(Brain – nuclei). In all cases, df = 56.

Nucleus	r^2	coef.	SE	95% Confidence Interval		int.	SEE
				lower	upper		
log(Telen)	0.846	1.083	0.062	0.981	1.212	0.094	0.080
log(HVC)	0.303	0.852	0.173	0.486	1.140	-2.447	0.248
log(RA)	0.517	0.930	0.120	0.688	1.170	-3.003	0.172
log(Area X)	0.476	0.917	0.129	0.668	1.146	-2.282	0.184
log(LMAN)	0.416	0.867	0.137	0.599	1.138	-3.372	0.197
log(MMAN)	0.339	0.816	0.152	0.540	1.122	-3.155	0.219
log(Uva)	0.728	0.690	0.056	0.584	0.802	-2.980	0.081
log(DM)	0.751	0.676	0.052	0.575	0.779	-2.913	0.075
log(nXIIts)	0.428	0.724	0.112	0.503	0.914	-3.291	0.161
log(Hp)	0.833	0.960	0.057	0.850	1.069	-1.656	0.082
log(Sep)	0.855	0.846	0.047	0.761	0.933	-1.952	0.067
log(TnA)	0.710	0.742	0.063	0.624	0.868	-2.078	0.091
log(MVL)	0.744	0.981	0.077	0.825	1.121	-3.218	0.110
log(E)	0.803	0.922	0.061	0.806	1.049	-1.799	0.088
log(Rt)	0.749	0.810	0.063	0.686	0.929	-2.043	0.090
log(Pt)	0.691	0.818	0.073	0.663	0.947	-3.405	0.105
log(SpL)	0.656	0.782	0.076	0.631	0.928	-2.793	0.109
log(SP)	0.620	0.702	0.073	0.555	0.837	-2.744	0.105
log(SLu)	0.751	0.840	0.065	0.704	0.960	-3.345	0.093
log(Ov)	0.790	0.817	0.056	0.716	0.931	-3.205	0.081
log(MLd)	0.604	0.630	0.068	0.506	0.757	-2.064	0.098
log(SON)	0.793	0.636	0.043	0.554	0.725	-2.954	0.062
log(NL)	0.731	0.602	0.049	0.505	0.695	-2.795	0.070
log(NM)	0.744	0.638	0.050	0.544	0.730	-2.806	0.072

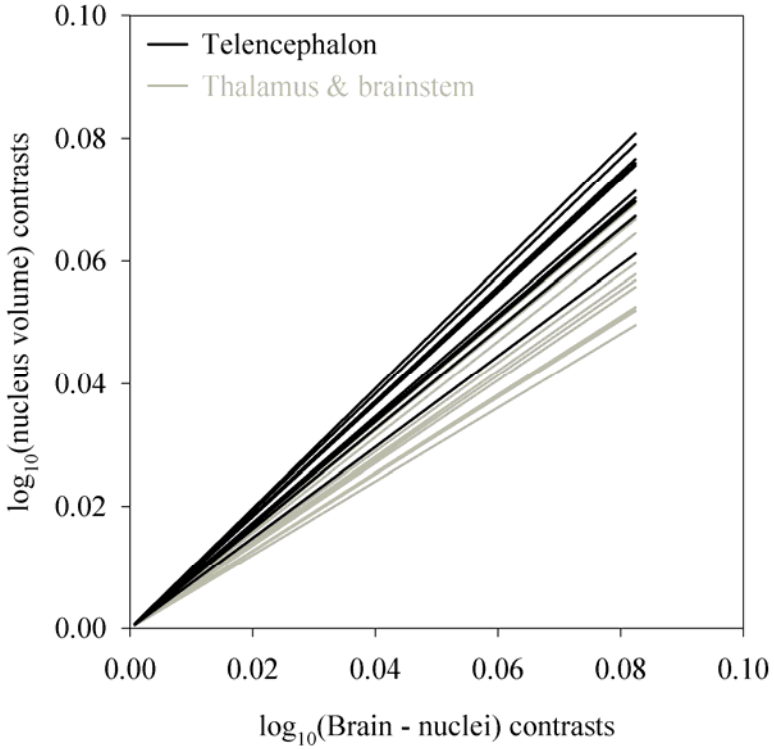


Figure 3.2. Nuclei within the telencephalon (black) have greater allometric slopes than those in the thalamus or brainstem (gray). Note that slopes from GLS models are identical to those from regressions of independent contrasts when the latter are forced through the origin.

Table 3.3. (*a*) First five eigenvalues and percent total variance explained from a factor analysis of independent contrasts of $\log_{10}$-transformed data. (*b*) Factor loadings for independent contrasts of each nucleus in a two-factor model.

Factor	Eigenvalue	% Variance
1	16.253	70.667
2	2.416	10.503
3	0.737	3.205
4	0.641	2.785
5	0.583	2.535
Nucleus	**Factor 1**	**Factor 2**
HVC	-0.099	0.952
RA	0.119	0.864
Area X	0.066	0.900
LMAN	0.052	0.847
MMAN	-0.024	0.849
Uva	0.543	0.448
DM	0.703	0.265
nXIIts	0.273	0.616
Hp	0.900	0.022
Sep	0.833	0.114
TnA	0.626	0.268
MVL	0.960	-0.083
E	1.021	-0.095
Rt	1.007	-0.094
Pt	1.015	-0.168
SpL	0.951	-0.061
SP	0.991	-0.154
SLu	0.945	-0.010
Ov	0.765	0.229
MLd	0.727	0.178
SON	0.805	0.172
NL	0.784	0.160
NM	0.746	0.239

The pronounced variability of the song system is further underscored by comparisons between species of similar size (Figure 3.3). For example, the spotted flycatcher (*Muscicapa striata*) has an HVC volume that is 15-fold larger than that of the common yellowthroat (*Geothlypis trichas*); the common starling (*Sturnus vulgaris*) has an RA volume that is 5-fold larger than that of the blue jay (*Cyanocitta cristata*); and the sand martin (*Riparia riparia*) has an LMAN volume that is nearly 5-fold larger than that of the black-capped chickadee (*Poecile atricapillus*) despite its substantially smaller brain volume. By comparison, maximum differences across other nuclei rarely exceeded a factor of 3. The relatively large Hp of the black-capped chickadee, for example, is only 2.8-times larger than that of the European goldfinch (*Carduelis carduelis*), which had one of the smallest relative Hp volumes measured.

(c) *Relationships between and within systems*

Relationships between systems were assessed by first adding the volumes of all nuclei within one system and then testing for correlations between them with size [i.e. log(brain – nuclei)] included as a confounding variable. After accounting for size, the volume of the tectofugal visual system was positively correlated with the limbic structures ($r = 0.30$, $p = 0.02$) and was nearly significantly related to the auditory system ($r = 0.25$, $p = 0.06$). In contrast, the volume of the song system was inversely correlated with the limbic structures ($r = -0.25$, $p = 0.04$) and was nearly so with the visual system ($r = -0.25$, $p = 0.06$). These associations involving the song system were investigated in more detail. Significant relationships existed between Hp and RA ($r = -0.43$, $p = 0.0008$), LMAN ($r = -0.28$, $p = 0.03$), and nXIIts ($r = -0.37$, $p = 0.005$) and between Sep and Area X ($r = -0.27$, $p = 0.04$) and nXIIts ($r = -0.37$, $p = 0.005$). They were also present between MVL and LMAN ($r = -0.30$, $p = 0.02$) and E and Area X ($r = -0.29$, $p = 0.03$).

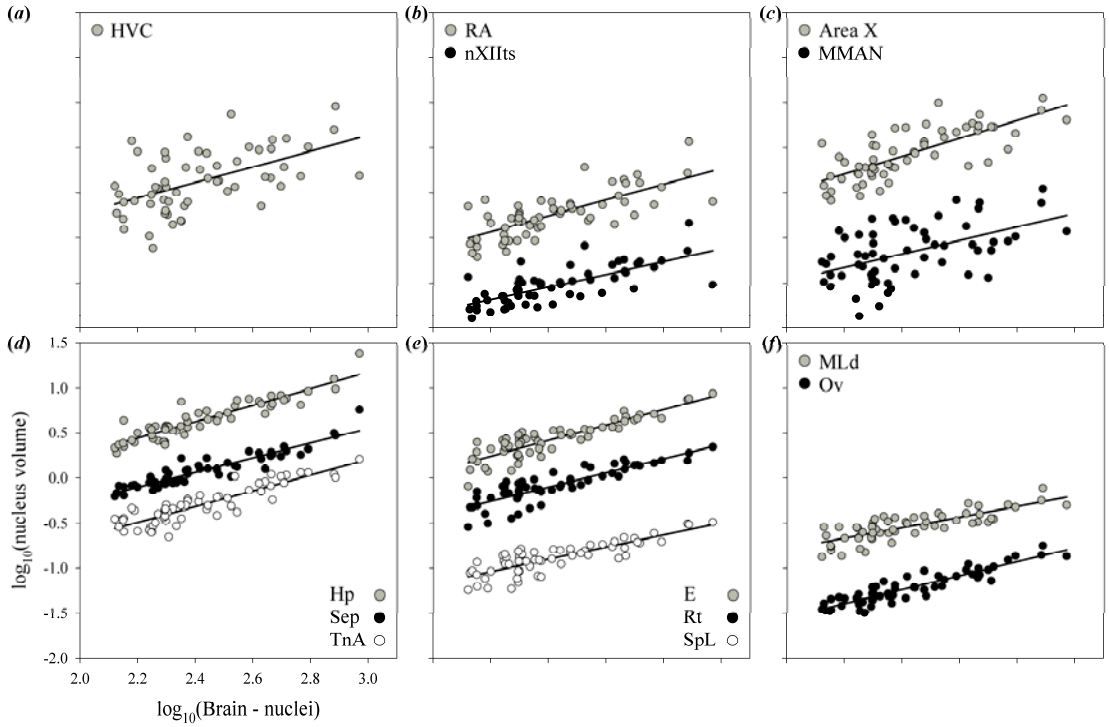


Figure 3.3. Variability in allometric relationships for several nuclei across systems. Note that the plots depict log-transformed data and not independent contrasts for illustrative purposes. Relative song nucleus volumes (*a –c*) were much more variable than those from the limbic (*d*), visual (*e*), or auditory (*f*) systems.

The volumes of nuclei within each system were positively and strongly correlated after accounting for size in most cases, and the strongest correlations were generally between directly connected areas (Tables 3.4-3.7). Significant associations existed between most song nuclei, including all telencephalic areas (all $p \leq 0.001$) and between those areas and both Uva and nXIIts (all $p \leq 0.01$). Nucleus DM was an exception and was not related to any areas but had nearly significant relationships with HVC and Uva ($0.05 \leq p \leq 0.09$). Among limbic structures, Hp and Sep were positively correlated with each other ($r = 0.50$, $p = 6.0 \times 10^{-5}$) but neither was related to TnA (both $p > 0.65$). All visual nuclei (all $p < 0.03$) and all but one pair of auditory nuclei (Ov and NM, $p = 0.09$; all other $p < 0.04$) were significantly correlated.

The cellular basis of these relationships was investigated by estimating neuron densities and numbers in 11 nuclei within the song, limbic, and visual systems. Densities in all 11 were inversely and strongly related to overall brain size (all $p < 0.0001$). For all areas except the Sep, relative neuron number was strongly and positively correlated with relative nucleus volume (Table 3.8; $0.54 \leq r^2 \leq 0.98$, all $p < 0.0001$) whereas neuron density was inversely related to relative volume for only 5 of these 10 (RA, MMAN, nXIIts, Hp, Rt; all $p < 0.03$). In each case, density explained a much smaller proportion of relative volume variation ($0.13 \leq r^2 \leq 0.36$) than did neuron number. The opposite was true for relative Sep volume. Here, a smaller amount of the variation in relative volume was explained by relative neuron number ($r^2 = 0.12$, $p = 0.048$) than neuron density ($r^2 = 0.27$, $p = 0.002$). Finally, most of the correlations between relative nucleus volumes also existed beween their relative neuron numbers, though these relationships did tend to lose strength (Tables 3.9 and 3.10). Two relationships lost statistical significance; relative neuron numbers were not correlated between HVC and nXIIts ($r = 0.21$, $p = 0.11$) nor between Hp and Sep ($r = 0.34$, $p = 0.051$).

Table 3.4. Regression matrices between the $\log_{10}$-transformed song nucleus volumes. The size factor log(brain – nuclei) was a confounding variable in each regression and statistics refer to the partial correlations. Directly connected nuclei are indicated by shaded cells. In all cases, df = 55.

		HVC	RA	Area X	LMAN	MMAN	Uva	DM
RA	*r*	**0.709**						
	t	**7.453**						
	p	**6.82E-10**						
Area X	*r*	**0.684**	**0.690**					
	t	**6.951**	**7.079**					
	p	**4.54E-09**	**2.81E-09**					
LMAN	*r*	**0.498**	**0.689**	**0.710**				
	t	**4.258**	**7.048**	**7.471**				
	p	**8.12E-05**	**3.15E-09**	**6.39E-10**				
MMAN	*r*	**0.744**	**0.459**	**0.570**	**0.394**			
	t	**8.269**	**3.832**	**5.144**	**3.178**			
	p	**3.18E-11**	**3.28E-04**	**3.70E-06**	**0.002**			
Uva	*r*	**0.340**	**0.420**	**0.451**	**0.408**	0.050		
	t	**2.679**	**3.436**	**3.744**	**3.311**	0.375		
	p	**0.010**	**0.001**	**4.36E-04**	**0.002**	0.709		
DM	*r*	0.241	0.168	0.181	0.073	0.151	0.233	
	t	1.841	1.266	1.363	0.544	1.130	1.778	
	p	0.071	0.211	0.179	0.588	0.264	0.081	
nXIIts	*r*	**0.332**	**0.621**	**0.496**	**0.497**	0.174	0.103	0.150
	t	**2.610**	**5.880**	**4.242**	**4.249**	1.308	0.770	1.127
	p	**0.012**	**2.52E-07**	**8.57E-05**	**8.37E-05**	0.196	0.444	0.265

Table 3.5. Regression matrices between the $\log_{10}$-transformed volumes of limbic structures. The size factor log(brain – nuclei) was a confounding variable in each regression and statistics refer to the partial correlations. Directly connected nuclei are indicated by shaded cells. In all cases, df = 55.

		Hp	**Sep**
Sep	*r*	**0.508**	
	t	**4.370**	
	p	**5.57E-05**	
TnA	*r*	0.019	-0.045
	t	0.141	-0.334
	p	0.888	0.739

Table 3.6. Regression matrices between the $\log_{10}$-transformed tectofugal visual nucleus volumes. The size factor log(brain – nuclei) was a confounding variable in each regression and statistics refer to the partial correlations. Directly connected nuclei are indicated by shaded cells. In all cases, df = 55.

		MVL	E	Rt	Pt	SpL	SP
E	*r*	**0.642**					
	t	**6.215**					
	p	**7.21E-08**					
Rt	*r*	**0.580**	**0.825**				
	t	**5.274**	**10.837**				
	p	**2.32E-06**	**2.90E-15**				
Pt	*r*	**0.464**	**0.718**	**0.703**			
	t	**3.889**	**7.658**	**7.322**			
	p	**2.74E-04**	**3.15E-10**	**1.12E-09**			
SpL	*r*	**0.306**	**0.514**	**0.483**	**0.557**		
	t	**2.381**	**4.445**	**4.088**	**4.975**		
	p	**0.021**	**4.30E-05**	**1.43E-04**	**6.76E-06**		
SP	*r*	**0.366**	**0.745**	**0.773**	**0.673**	**0.531**	
	t	**2.912**	**8.289**	**9.026**	**6.740**	**4.650**	
	p	**0.005**	**2.96E-11**	**1.92E-12**	**1.00E-08**	**2.12E-05**	
SLu	*r*	**0.304**	**0.523**	**0.621**	**0.513**	**0.558**	**0.555**
	t	**2.365**	**4.553**	**5.869**	**4.435**	**4.989**	**4.946**
	p	**0.022**	**2.97E-05**	**2.61E-07**	**4.46E-05**	**6.44E-06**	**7.49E-06**

Table 3.7. Regression matrices between the $\log_{10}$-transformed auditory nucleus volumes. The size factor log(brain – nuclei) was a confounding variable in each regression and statistics refer to the partial correlations. Directly connected nuclei are indicated by shaded cells. In all cases, df = 55.

		Ov	MLd	SON	NL
MLd	*r*	**0.588**			
	t	**5.387**			
	p	**1.53E-06**			
SON	*r*	**0.321**	**0.413**		
	t	**2.513**	**3.368**		
	p	**0.015**	**0.001**		
NL	*r*	**0.273**	**0.493**	**0.518**	
	t	**2.106**	**4.202**	**4.489**	
	p	**0.040**	**9.79E-05**	**3.71E-05**	
NM	*r*	0.232	**0.438**	**0.385**	**0.600**
	t	1.771	**3.613**	**3.093**	**5.556**
	p	0.082	**0.001**	**0.003**	**8.29E-07**

Table 3.8. Regressions between $\log_{10}$-transformed nucleus volumes and neuron number or density of that nucleus. The size factor log(brain – nuclei) was a confounding variable in each regression and statistics refer to the partial correlations.

Volume		neuron #	neuron density
HVC	r^2	**0.977**	0.037
	t	**48.695**	-1.469
	p	**<0.0001**	0.147
RA	r^2	**0.873**	**0.305**
	t	**19.638**	**-4.956**
	p	**<0.0001**	**<0.0001**
LMAN	r^2	**0.875**	0.059
	t	**16.545**	-1.543
	p	**<0.0001**	0.131
MMAN	r^2	**0.901**	**0.134**
	t	**18.800**	**-2.457**
	p	**<0.0001**	**0.019**
nXIIts	r^2	**0.734**	**0.257**
	t	**12.427**	**-4.406**
	p	**<0.0001**	**<0.0001**
Hp	r^2	**0.605**	**0.355**
	t	**7.003**	**-4.131**
	p	**<0.0001**	**3.00E-04**
Sep	r^2	**0.117**	**0.268**
	t	**2.060**	**-3.422**
	p	**0.048**	**0.002**
E	r^2	**0.594**	0.049
	t	**6.161**	-1.163
	p	**<0.0001**	0.255
Rt	r^2	**0.544**	**0.162**
	t	**5.573**	**-2.238**
	p	**<0.0001**	**0.034**
SpL	r^2	**0.663**	0.022
	t	**7.152**	0.761
	p	**<0.0001**	0.454
SP	r^2	**0.713**	0.020
	t	**8.040**	-0.729
	p	**<0.0001**	0.473

Table 3.9. Regression matrix of $\log_{10}$-transformed neuron numbers between various song nuclei. The size factor log(brain – nuclei) was a confounding variable in each regression and statistics refer to the partial correlations.

		HVC n#	RA n#	LMAN n#	MMAN n#
RA n#	*r*	**0.612**			
	t	**5.796**			
	p	**<0.0001**			
LMAN n#	*r*	**0.488**	**0.662**		
	t	**3.494**	**5.517**		
	p	**0.001**	**<0.0001**		
MMAN n#	*r*	**0.785**	**0.470**	**0.488**	
	t	**7.925**	**3.327**	**3.492**	
	p	**<0.0001**	**0.002**	**0.001**	
nXIIts n#	*r*	0.214	**0.533**	**0.608**	0.177
	t	1.638	**4.710**	**4.780**	1.126
	p	0.107	**<0.0001**	**<0.0001**	0.267

Table 3.10. Regression matrix of $\log_{10}$-transformed neuron numbers between various visual nuclei. The size factor log(brain – nuclei) was a confounding variable in each regression and statistics refer to the partial correlations.

		E n#	Rt n#	SpL n#
Rt n#	*r*	**0.657**		
	t	**4.441**		
	p	**1.00E-04**		
SpL n#	*r*	**0.604**	**0.449**	
	t	**3.867**	**2.560**	
	p	**0.001**	**0.017**	
SP n#	*r*	**0.569**	**0.563**	**0.573**
	t	**3.529**	**3.473**	**3.562**
	p	**0.002**	**0.002**	**0.001**

Discussion

The evolution of the avian brain has resembled that of mammals in several respects, and the data presented here reveal patterns that support both concerted and mosaic models of evolution. First, graceful scaling of brain subdivisions is present across species. The telencephalon exhibits hyperallometry relative to the rest of the brain and telencephalic nuclei have greater allometric slopes than those in the thalamus and brainstem. This is consistent with scaling patterns in mammals, in which the evolution of large brains has been suggested to result from a gradual stretching of conserved neurogenetic schedules (Finlay & Darlington 1995). While changes in the timing of neurogenesis do not entirely account for the large telencephalic fractions of songbirds, parrots, and waterfowl compared to other taxa (Striedter & Charvet 2008; Charvet & Striedter 2009a,b), it may still underlie the gradual differences within oscines. In the songbird, the telencephalic fraction is roughly equivalent to the tectum and medulla early in embryogenesis but expands disproportionately thereafter because its neural progenitors divide for longer periods of time (Charvet & Striedter 2009a). This growth appears to occur in two waves, one *in ovo* when new neurons are born throughout the entire brain and another after hatching, when they are almost exclusively added to the telencephalon (Alvarez-Buylla et al. 1994; Charvet & Striedter 2009a). Thus, gradually stretching developmental schedules as brain size increases, especially that which occurs after hatching, would disproportionately expand the number of telencephalic progenitors and could cause the gradual increase in telencephalic fraction observed here.

Second, the relative volumes of connected nuclei are strongly correlated and these associations are primarily due to coordinated changes in cell number. Similar relationships are present between the volumes of large brain regions in both mammals and birds (Barton & Harvey 2000; Whiting & Barton 2003; Iwaniuk et al. 2004). The

relative contributions of neuron number and density to relative nucleus volume generally correspond to the degree of convergent input each receives from its afferent source(s). For areas that are larger or comparable in size to their afferents, such as HVC, E, and SpL, relative volume accurately reflects neuron number and is unrelated to neuron density. By contrast, the relative volumes of nuclei that are densely innervated by a larger afferent source, such as RA, nXIIts, Sep, and Rt, also reflect differences in neuron density. This effect is presumably the result of increases in the number of axons and perhaps trophic-induced cell growth. As a consequence, interspecific differences in the relative volumes of nuclei that receive such dense innervation seem prone to overestimate the true differences in neuronal composition.

Coordinated changes in neuron number are perhaps to be expected if basic circuit organization and physiological mechanisms are conserved within orders. The developmental mechanisms that produce these correlations are less clear. At one extreme, brain development could be mosaic in that alterations in the regional boundaries, progenitor cell cycle rate, and/or timing of neurogenesis could be independently regulated in individual nuclei. Alternatively, cell proliferation and neurogenesis schedules may be broadly specified within larger subdivisions or regions, and new neurons could be differentially recruited into various nuclei as overall brain size changes. In either case, selection may then favor phenotypes in which nuclei comprising a functional pathway have changed together. It seems likely that activity-dependent mechanisms and/or epigenetic cascades also serve some role in this coordination given that correlations exist between widely distributed nuclei and that numerous molecular pathways are involved in neural tube regional specification (Garcia-Lopez et al. 2009). More descriptions of developmental differences between large and small species are needed to test these possibilities. It is clear, however, that correlations between connected areas cannot distinguish between them and that these

associations can exist within the context of scaling differences between subdivisions; therefore they do not, by themselves, provide clear evidence for mosaic brain evolution.

The most striking feature of this data set is the immense variability of the song system compared to other pathways. This is most obvious in nuclei HVC and MMAN, but even the relative volumes of RA, Area X, LMAN, and nXIIts vary by nearly twice as much as all other nuclei measured. To be sure, direct comparisons across nuclei are difficult because they differ in their connectivity, intrinsic circuitry, and physiology, all of which will influence their structure through evolution. Nevertheless, we are struck by the fact that nuclei within the three other pathways exhibit similar amounts of variation despite these differences and that they are all consistently and drastically less variable than the major song nuclei. This does not appear to reflect a lack of consequences when elaborated; hypertrophy of the hippocampus is associated with enhanced spatial memory (Krebs et al. 1989) and enlarged auditory brainstem nuclei are related to superior sound localization and perceptual abilities (Kubke et al. 2004). Interestingly, the interspecific variability in these three systems largely conforms to the ~2.5-fold differences allowed by the model of Finlay and Darlington (1995). Such uniformity clearly does not apply to the song system, however, which contains several nuclei that exhibit 5-fold or greater differences between species of similar size. Thus, the song system exhibits a degree of variation indicative of mosaic evolution, especially given that very large volumetric differences have emerged multiple times within the Passerida parvorder, in some cases quite rapidly (e.g., Sylviidae).

At first glance, this extreme variability of the song system would seem to invalidate the hypothesis that conserved developmental programs can be very influential in the evolution of the oscine brain. Yet, the development of the song

system is decidedly unusual. Neurons are incorporated into several song nuclei after surrounding tissues have been established, and this delay may effectively release these structures from otherwise powerful constraints and/or create opportunities for their selective alteration independently from other brain structures (Alvarez-Buylla et al. 1994). It is also interesting that the most variable nucleus measured, HVC, is one of the last to develop (e.g., Kirn & DeVoogd 1989; Nordeen et al. 1989). These observations are consistent with the notion that the sequence of brain development makes some changes more likely than others, and they suggest that later-developing nuclei may be more plastic. On the other hand, the late development and marked variability of the song system may be coincidental or related to other factors. The extreme variability of the song system may simply reflect more varied selection pressures on song, be they sexual or natural, than on other behaviors. It could also be a consequence of distinct physiological mechanisms between circuits, akin to a functional incompatibility between memory systems (Sherry & Schacter 1987). HVC neurons encode birdsong by means of a sparse code where individual neurons are specifically dedicated to the production of a single, brief sound (Hahnloser et al. 2002); incidentally, the elaboration of song repertoires is associated with dramatic increases in HVC neuron number (DeVoogd et al. 1993; Moore et al. 2009). The behavioral capacities associated with the other systems measured may not be encoded in such a way and can perhaps be effectively altered by more subtle changes, such as to intrinsic connectivity, synaptic weights, or internal chemistry (Goodson et al. 2006).

These data also highlight tendencies for 'discrete' systems to evolve in concert. For example, we have generally treated the visual and auditory systems as being functionally independent, though cross-modal integration certainly occurs and the degree of specialization in one sensory system may depend on the other. Many mammals, for instance, localize sounds for the purpose of visually fixating a source,

and sound localization acuity is related to best visual field width (Heffner & Heffner 1992). Similarly, enhanced perceptual systems that permit better visual pattern recognition (Watanabe et al. 2008) or auditory perception (Kubke et al. 2004) could aid in tasks that employ limbic structures, such as episodic memory (Hp; Clayton et al. 2003), territoriality or sociality (Sep; Goodson et al. 1999), and mating behaviors (TnA; Thompson et al. 1998; Svec et al. 2009). Hints at such relationships are reported here: limbic structures are positively correlated with visual nuclei and are nearly significantly related with auditory nuclei. Interestingly, the two negative trends observed both involved the song system, a circuit that underlies production of a reproductive advertisement signal that must somehow be costly to the senders (Zahavi 1975b; Bradbury & Vehrencamp 1998). At the level of individual nuclei, relative RA and nXIIts volumes are inversely correlated with the limbic system. Trade-offs will inevitably occur when comparing the relative sizes of various structures because a single brain cannot have all components that are relatively large or small. Yet, these would seem unlikely to be artifactual given that each occupies a small proportion of the brain. If these relationships genuinely exist, expansions of the song system could provide a means to advertise this neural trade-off. The exact location of arcopallial precursors in the neural tube is not known, but the dorsal ventricular ridge (meso-, nido-, and arcopallium) arises from lateral and ventral pallium, which are adjacent to the medial pallium that generates hippocampal neurons (Garcia-Lopez et al. 2009). Thus, a trade-off between the arcopallium and hippocampus may exist similar to that between neocortex and limbic structures in some orders of mammals (Reep et al. 2007).

In conclusion, this study documents the correlated evolution of functional neural circuits and provides evidence in support of both the concerted and mosaic models of evolution. The song system is dramatically more variable than others

measured, which may be related to its late development. Future work on interspecific differences in brain development will provide valuable insights into its role in the evolution of brain structure.

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CHAPTER 4

THE CAUDOMEDIAL NIDOPALLIUM (NCM) IS NECESSARY FOR MATE SONG PREFERENCES IN FEMALE ZEBRA FINCHES (*TAENIOPYGIA GUTTATA*)

Abstract

Birdsong is a sexually selected communication signal that functions in reproduction. Females actively attend to male song and can exhibit at least two general types of song preferences, one for familiar songs and another for high quality songs. The neural bases of these preferences are poorly understood. The present study sought to determine whether the caudomedial nidopallium (NCM), a region analogous to mammalian auditory association cortex, is necessary for the expression of these two types of preferences in adult female zebra finches (*Taeniopygia guttata*). NCM was bilaterally and reversibly inactivated with tetrodotoxin and subjects' preferences for their mate's song over a novel song and for complex 'tutored' song over abnormally simple 'isolate' song were tested using a phonotaxis task. Inactivation of NCM eliminated the mate song preference of most subjects but had a much more modest effect on tutored song preferences. Inactivation of the caudal mesopallium (CM) had no effect on mate song preferences. These results demonstrate for the first time that NCM is required for the expression of song preferences by adult female songbirds. Potential pathways underlying this behavior and functional compartmentalization of the auditory forebrain are discussed.

Introduction

Birdsong is a learned vocal communication signal that functions in reproduction. While a great deal is known about the neural mechanisms underlying male song learning and production, much less is understood about the neural bases of

song perception. In particular, the specific functions of auditory forebrain areas have not been unambiguously identified. Female songbirds provide opportunities to investigate these issues because they are often the intended audience of male song and they actively attend to it. They can make acute discriminations between songs (Miller 1979a; Searcy & Brenowitz 1988; Riebel & Smallegange 2003) and sophisticated judgments about male quality based on song (Kroodsma 1976; Hasselquist et al. 1996; Nowicki et al. 2002). The present study seeks to identify areas underlying these complex abilities.

Female zebra finches (*Taeniopygia guttata*) exhibit at least two general types of song preferences. One is based on song familiarity; they prefer both their father's and mate's songs over those of unfamiliar males and these persist over long time periods, indicating that they form stable memories of specific songs (Miller 1979a,b; Riebel 2000). The second is based on song quality, which may be a result of song content or performance-based aspects. In the case of the former, females prefer longer songs comprised of more syllables (Neubauer 1999a) and songs that were faithfully copied from an adult over those improvised by males raised in isolation (Lauay et al. 2004). For the latter, they prefer higher song rates (Houtman 1992) as well as female-directed songs, which are slightly faster and more stereotyped than undirected songs (Woolley & Doupe 2008). The neural bases of these two types of preferences are unknown, but they seem likely to involve different brain regions given that the first is based on a specific auditory memory and is not necessarily dependent on one song's perceived quality relative to another, while the second can be shown with songs the bird has never heard.

The caudomedial nidopallium (NCM) appears to be especially important for the encoding of song-related memories. It receives highly processed auditory information from Field L, the avian analogue of mammalian auditory cortex, is

densely interconnected with other regions in the auditory forebrain, and is specifically activated by playback of conspecific song (Figure 4.1; Mello et al. 1992; Vates et al. 1996). Both physiological and immediate early gene (IEG) responses habituate to repeated presentations of a stimulus (Chew et al. 1995; Mello et al. 1995); this effect is specific to individual songs and is long-lasting, suggesting that that NCM has the capacity for storing many distinct auditory memories (Chew et al. 1996; Stripling et al. 1997). Memory of the tutor song may be stored here, for instance, as both IEG levels and physiological habituation rates following tutor song playback are related to the strength of song learning in males (Bolhuis et al. 2000; Phan et al. 2006). Similarly, ZENK protein levels are habituated in females following playback of the mate's song compared to a novel song (Woolley & Doupe 2008) and are elevated following the acquisition of new song discriminations (Gentner et al. 2004).

Whether NCM also serves a role in song quality assessment is less clear. ZENK levels in female zebra finches are equivalent following playback of directed or undirected songs (Woolley & Doupe 2008). Yet, they are elevated in female European starlings (*Sturnus vulgaris*) following playback of longer songs, which they find more attractive (Gentner & Hulse 2000; Gentner et al. 2001), and in female white-crowned sparrows (*Zonotrichia leucophrys*) following playback of a more attractive local versus foreign dialect (Maney et al. 2003). Each of these latter findings may reflect song salience, however, because they both depend on previous experience (Sockman et al. 2002). The goal of this study was to ascertain whether NCM is necessary for these two types of song preferences in female adult zebra finches.

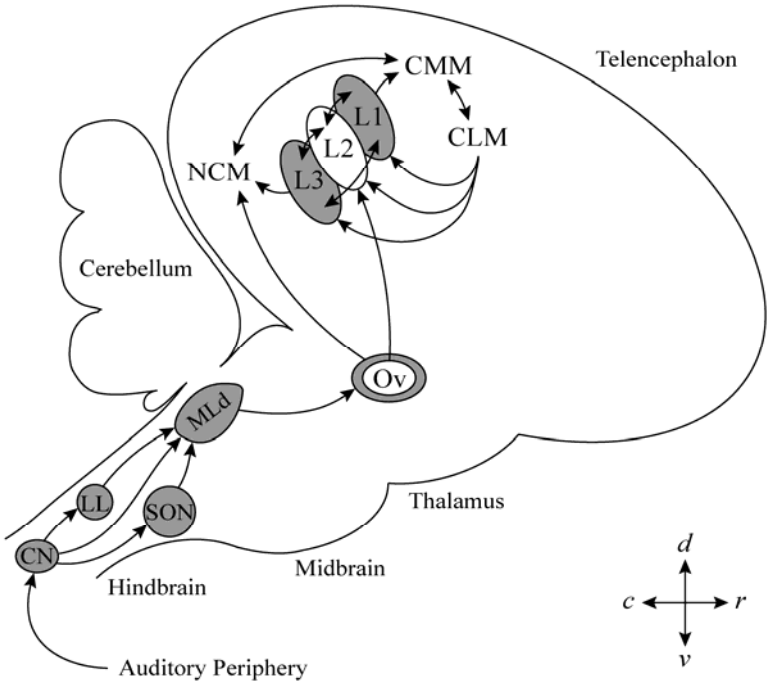

Figure 4.1. Diagram of the ascending auditory pathway and major auditory forebrain areas. Cannulae targeted NCM and a central region of CM that was slightly lateral to CMM.

Methods

(a) *Subjects*

Subjects were 16 adult female zebra finches (≥240 days old) bred from an in-house colony. Prior to mate song preference testing, each female was housed with an adult male in an individual cage (45×40×40 cm) outfit with a nest box and nesting material for at least 3 months. All subjects had successfully reared offspring to adulthood before testing. Before assessing preferences for tutored versus isolate songs, subjects were housed individually without a nest box. Four birds with cannulae directed at NCM were tested in both the song familiarity and song quality preference tests. Cages occupied two rooms; opaque sheets visually isolated all cages within a room and birds housed in one room never heard the songs of males in the other except during behavioral tests (see below). Birds were kept on a 14h:10h light:dark schedule at all times and food and water were available *ad libitum*.

(b) *Cannulation Surgery*

All surgical procedures were approved by the Cornell University Institutional Animal Care and Use Committee. Birds were anesthetized with an intramuscular injection of Equithesin (0.006 ml · g^{-1}) and placed in a stereotaxic apparatus outfitted with a custom beak bar. Stainless steel guide cannulae (27 ga., Small Parts, Inc.) cut to 4 mm were bilaterally placed over NCM (0.6 mm rostral and 0.5 mm lateral of sagittal sinus bifurcation, 0.3 mm ventral) or CM (2.0 mm rostral, 1.0 mm lateral, 0.1 mm ventral) and affixed to the skull using dental cement. Following surgery, small wire plugs were placed in the cannulae to prevent clogging and birds were administered the analgesic butorphanol (2.5×10^{-4} mg · g^{-1}). Birds were placed in a small recovery cage with a heating pad until they were able to perch normally and then returned to their home cage. They were allowed to recover for 1 week before behavioral testing.

(c) *Song recordings*

Male songs were recorded (44.1 kHz, 16-bit) in a soundproof chamber with a Marantz PMD670 digital audio recorder and Sennheiser ME62 omnidirectional microphone with parabolic reflector. Playback clips of female-directed songs were created using Syrinx (Burt 2006). Each clip contained a single song bout with 3-5 introductory notes and 4 motifs per bout. Bouts were normalized for peak amplitude but were otherwise unmanipulated. In cases where the song bout selected to create the playback clip contained more than 4 consecutive motifs, the first 4 were used. Stimuli were fed through a Pioneer SA-410 amplifier, and maximum song amplitude was 70 dB SPL on the near perch of the testing cage.

(d) *Drug Infusions & Behavioral Testing*

The behavioral testing cage consisted of three connected cages (135×40×40 cm) to form two approach zones and a central, neutral zone that contained a nest box with nesting material and food and water (Figure 4.2a). Passageways were visually emphasized with a white border to promote free movement throughout the entire cage. Speakers (Morel MDT29) with flat frequency responses (0.5 – 10 kHz) were placed at opposite ends of the cage and male zebra finch models affixed to perches on the front of each speaker stand. A white semi-sheer fabric was draped over each end of the cage to obscure fine differences in physical appearance between the models and the subjects' mates. Four symmetrically-positioned perches traversed the width of the cage: two within the neutral cage and one in each approach zone located 12 cm from the cage edge and 16 cm from the cage floor. Infrared emitters and sensors (Digi-Key) were aimed over the end perches and read with a National Instruments USB-6008 data acquisition card and custom Matlab software to enable the automated recording of bird position at these two locations.

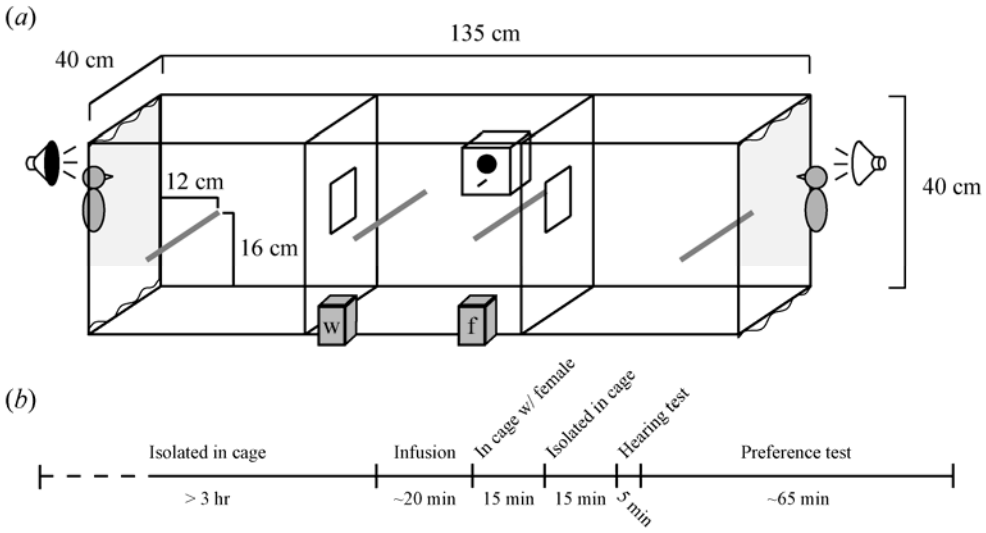

Figure 4.2. (*a*) Diagram of the phonotaxis testing cage with a central, neutral zone and two approach zones. Cage ends were draped with a white semi-sheer fabric to obscure details of the male models. w: water, f: food. (*b*) Timeline for the infusion, re-acclimation period, hearing test, and song preference test.

A timeline of the experimental procedure is shown in Figure 4.2b. Prior to testing, subjects were isolated in the testing cage for at least 3 hours. To administer drug infusions, they were restrained in a custom cloth jacket and placed in a body mold with a head strap. Subjects were infused bilaterally with 0.15 µl of 0.9% saline for control trials and with 0.15 µl of 10 µM tetrodotoxin (TTX; Sigma), a potent Na^+ channel blocker, dissolved in saline for experimental trials. Infusions were administered with a 0.5 µl Hamilton syringe and 32-gauge needle. Polyethylene tubing covered the entire needle except 5 mm from the tip to control the needle's depth (i.e. 1 mm past the base of the guide cannula). Infusion rate was ≤0.03 µl/min to prevent permanent tissue damage and the needle was left in place for at least 3 additional minutes before removal to allow drug diffusion. In total, infusions lasted approximately 20 minutes.

Following infusions, subjects were allowed to re-acclimate to the testing cage for 30 minutes. A second female was placed in the cage for companionship during the first 15 minutes, which helped birds recover from any distress caused by handling, and the subject was again isolated for the last 15 minutes. Next, a hearing test was conducted because both NCM and CM are immediately adjacent to Field L, the avian analogue of primary auditory cortex, and we wished to rule out the possibility that drug-induced changes in behavior resulted from deafening the bird. Recorded aviary noises were played through both loudspeakers (53 dB SPL in the central cage) and the number of distance (or long) calls given by the subject was recorded. Distance calls are conspicuously long (~0.4 sec) and loud vocalizations frequently given by birds isolated from a flock (Zann 1984). This test began with 5 minutes of silence to obtain the bird's baseline calling rate, and then a 30 sec clip of aviary sounds was followed by 30 sec of silence; the number of calls given during this one-minute interval was

recorded. Some subjects (n = 5) were also presented with a 15 sec clip of white noise and/or a 3 kHz tone to assess whether calling was general to all auditory stimuli.

Female song preferences were measured in two ways: trials began with a two-choice phonotaxis paradigm (passive phase, ~35 min) and were immediately followed by a two-choice operant paradigm (active phase, 30 min) in which song playback was the reward. The passive phase began with the left speaker broadcasting 4 song bouts with 9 sec separating the onset of each bout (4 motifs per bout, each bout lasted ~5-7 sec) and a 15-sec silent interval followed playback of the fourth bout. This routine was then initiated from the right speaker. The total cycle (left and right playbacks) was repeated 20 times. Speaker approach times were recorded as the amount of time the subject spent on the left and right end perches. During the active phase, the subject controlled song playback with its position. Perching on either end elicited playback of one bout from that speaker, and a 5-sec silent interval separated each bout if the bird remained on a single perch for extended periods.

Trials were only included in the analyses if the subjects met one (passive) or two (active) criteria. For the passive phase, subjects were required to spend at least 10% of the total time on either end perch. For the active phase, subjects were required to satisfy the passive phase criterion and also spend 10% of this trial on either end perch. This occurred in all but two cases (the same bird), therefore no active phase data are presented for that bird. Birds were tested until they met these criteria for a total of 6 preference trials for song familiarity (mate vs. stranger) or song quality (tutored vs. isolate). In each case, two control (saline) trials preceded two experimental (TTX) trials which were followed by two additional control trials. For each treatment, the speaker associated with a particular song was switched between the two trials to control for potential side preferences. Trials were separated by at least three days, during which time the subject was returned to its home cage.

The song familiarity preference test paired the song of the subject's mate with that of a stranger matched for syllable repertoire, and each trial used a novel stranger song. Songs of males housed in the same room as the subject were never used as a stranger song; however, to control for the possibility that mate songs were inherently more attractive than stranger songs, those of males housed in the other room (and mated with other female subjects) were used as stranger songs whenever possible. For the song quality preference tests, one tutored song (i.e. of a male that faithfully copied the song of a normal adult) was paired with one 'isolate' song (i.e. of a male that improvised its song because it was raised in isolation from adults). Isolate songs usually contain fewer syllables, are less spectrotemporally complex, and occasionally contain abnormal features such as slow, upward frequency modulations (Figure 4.3). The same song pair was used in all six trials for a given subject but different subjects were presented with different song pairs. Here, songs were chosen to maximize the disparity in quality, so that tutored songs typically had more syllables and were slightly longer in duration and isolate songs were especially simple. Even still, not all females consistently preferred tutored songs in initial saline trials; only those that did were used in subsequent tests.

Female discriminatory abilities were also measured from recordings made during the passive phase of song preference trials. Both call latency relative to bout onset, which is related to song familiarity (Stripling et al. 2003), and call number during each bout were measured. Measurements did not differentiate between contact (short) and distance (long) calls. The same measurements were made from initial saline trials of song quality preference tests, but no differences in call number or latency to tutored or isolate songs existed, therefore recordings from subsequent trials were not analyzed.

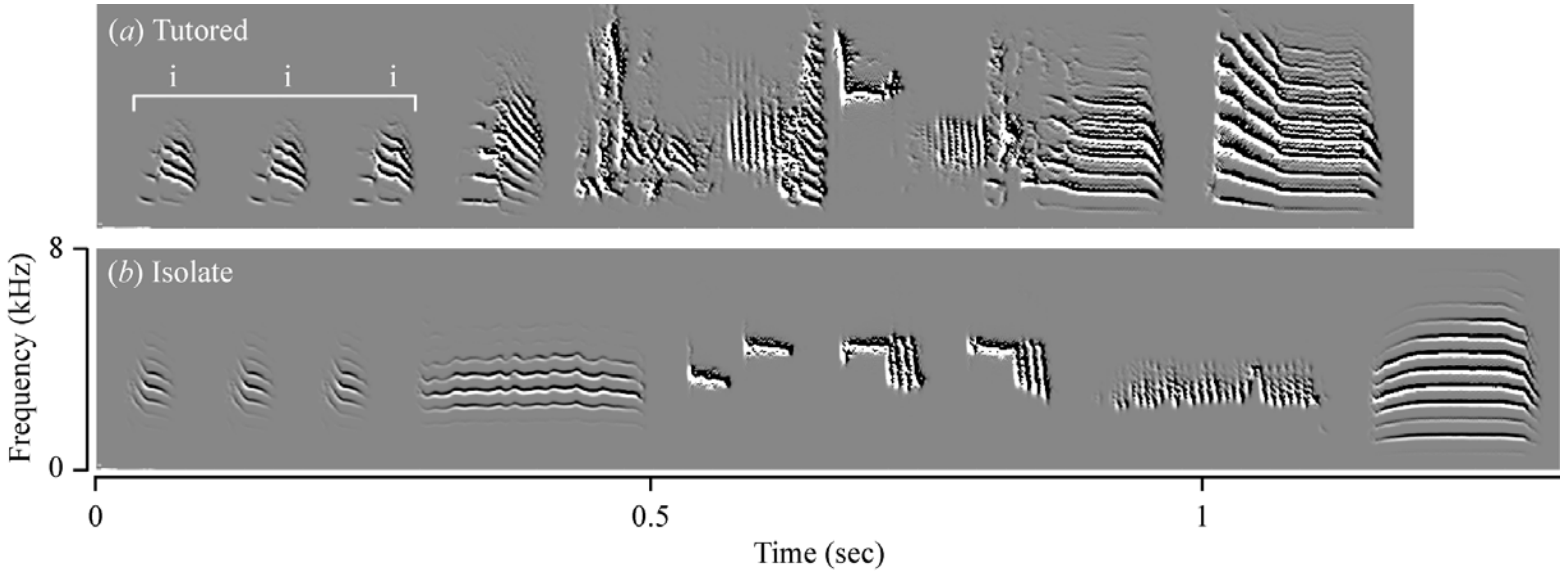

Figure 4.3. Spectrograms of a single motif from a representative (*a*) normal, tutored song and (*b*) isolate song. Each playback clip was comprised of a bout containing 3-5 introductory notes (i) and 4 motifs. In the isolate song, note the lack of syllables with complex spectrotemporal modulations and the abnormal, slow upward frequency modulation of the last syllable.

The effectiveness of the TTX was periodically assessed using male zebra finches. Two males had cannulae bilaterally implanted targeting HVC, a song premotor nucleus located in the dorsal nidopallium, and were housed individually thereafter. Approximately every two to three weeks, each was infused with the same volume and concentration of TTX as administered to the females, allowed to recover in their home cage for ~30 minutes, and then presented with a female. The males reliably approached the female and attempted to sing (as judged by their posture and production of introductory notes), but they failed to do so following TTX infusions. New drug dilutions were made from a stock solution whenever this effect did not last >2h.

(e) *Histology*

Following behavioral testing, cannula placement was verified by infusing 0.15 µl of 1 mM BODIPY-muscimol (Molecular Probes, MW=607.46) dissolved in saline and DMSO. Birds were infused as described above, then administered an overdose of barbiturate anesthetic and transcardially perfused with 0.9% saline followed by 10% formalin in saline. Brains were post-fixed for 24h, cryoprotected in 30% sucrose/10% formalin for 24h, embedded in gelatin and cryoprotected for at least 5 days. Brains were sectioned at 40 µm in the parasagittal plane on a freezing, sliding microtome; alternate sections were mounted on gel-coated slides and coverslipped immediately with ProLong Gold with DAPI (Molecular Probes) or allowed to dry and Nissl-stained with cresyl violet. Needle track locations were measured in Nissl-stained sections with aid of a camera lucida; approximate drug spread was estimated from digital images of the BODIPY-muscimol. The spread of BODIPY-muscimol likely under-represented the spread of TTX due to its slightly larger size and greater hydrophobicity, but it nevertheless provided visual confirmation of successful infusions.

(f) *Data Analysis*

Following previous studies, we reserved the term 'preference' for instances when a subject spent at least twice as much time on one side compared to the other (e.g., Miller 1979a; Woolley & Doupe 2008). The passive and active phases of the phonotaxis task were analyzed separately. Data were represented as the time spent on the mate or tutored song side as a proportion of time spent on either end perch [e.g., mate/(mate+stranger)]; this was viewed as the most representative index of relative preference strength because it factored out time spent in the middle cage or on the cage floor. Each trial was given equal weight in final analyses by first computing the preference scores or call data and then averaging the two from a given treatment. These averages were analyzed with a two-way repeated measures ANOVA with 'treatment' and 'mate speaker' (i.e. left or right) as factors. The Holm-Sidák Pairwise test was used for *post hoc* comparisons.

Results

(a) *General activity levels*

All birds called during all hearing tests following both saline and TTX infusions (Figure 4.4). Within each treatment, the number of distance calls during and immediately after aviary noise playbacks was greater than that during the preceding five minutes of silence (all $p < 0.01$). There were no differences between saline and TTX treatments ($p = 0.48$). None of the five birds presented with white noise and/or 3-kHz tones called during these stimuli following any treatment, indicating that they retained the ability to discriminate complex, species-typical sounds from simpler stimuli.

There were no significant differences in the proportion of total trial time spent on either end perch across the three treatments (i.e. Saline 1, TTX, Saline 2). For mate vs. novel song tests following NCM infusions, birds spent 42±16% (mean ± sd; p =

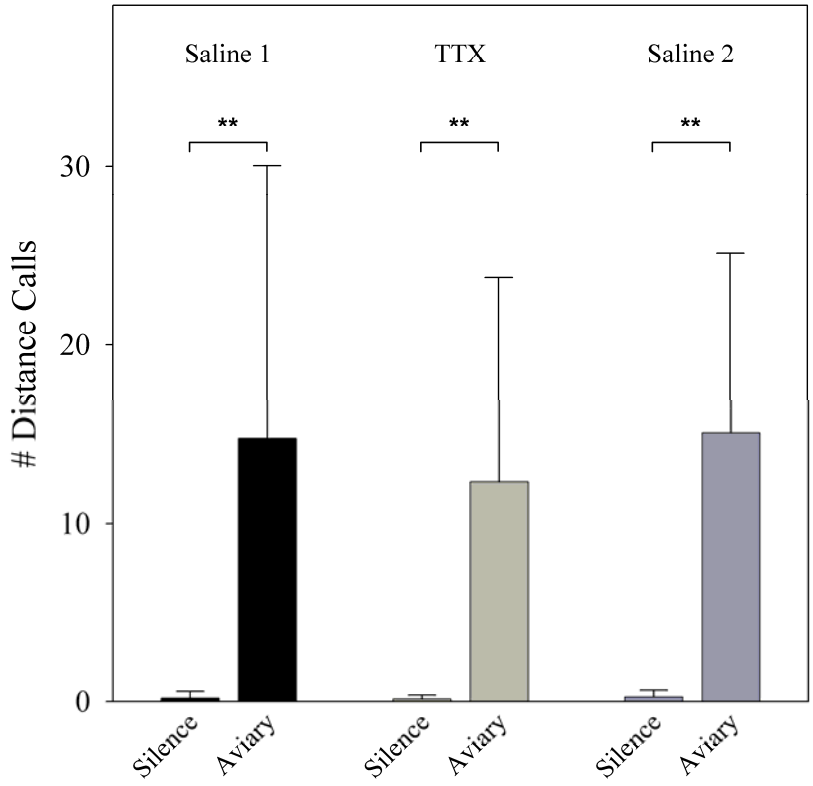

Figure 4.4. Results from hearing tests conducted before both types of song preference tests. All birds called during each hearing test, and the average number of calls to aviary noise playback was significantly greater than those produced during the preceding 5 min of silence for each treatment (all $p < 0.01$). The average number of calls did not differ across treatments ($p = 0.48$).

0.22) of the passive phase and 37±21% ($p = 0.86$) of the active phase on the end perches. For the tutored vs. isolate song tests, they spent 51±22% ($p = 0.91$) of the passive phase and 46±27% ($p = 0.06$) of the active phase on the end perches. Finally, in the mate-novel tests following CM infusions the birds spent 45±22% ($p = 0.50$) of the passive phase and 28±19% ($p = 0.17$) of the active phase on the end perches.

(b) *NCM inactivation*

All birds exhibited strong preferences for their mate's song over novel songs in both the passive and active phases following saline infusions into NCM (Figure 4.5). During the passive phase, nearly all of the time that subjects spent on an end perch in Saline 1 trials was next to the mate song speaker (0.98±0.04, mean ± sd). This preference was reduced following TTX infusions (0.57±0.25, $p < 0.001$) and was abolished in 5 out of 8 birds, one of which reversed its preference and strongly preferred the novel song in both trials. These effects were reversible, as mate song preferences were universally restored during Saline 2 trials (0.88±0.09; $p = 0.001$). The reduced mate song preferences in TTX trials remained significant after exclusion of the bird that preferred the novel song (both $p < 0.001$). The effects of NCM inactivation were even stronger in the active phase. Again, all birds strongly preferred their mate's song in both Saline 1 (0.99±0.01) and Saline 2 (0.91±0.07) trials, but this preference was eliminated in all birds following administration of TTX (0.31±0.23; both $p < 0.001$). One bird did not move to an end perch during the active phase of either experimental trial, thus $n = 7$ for this task. The birds did not exhibit a side preference across trials and treatments in either phase (both $p > 0.55$).

Mirroring the phonotaxis data, females called more quickly to playback of their mate's song than to novel songs during both saline trials (Wilcoxon signed rank test, both $p < 0.03$) but not during the TTX trials ($p = 0.11$; Figure 4.6a). However, the differences in ratios between treatments were not significant ($p = 0.10$) because the

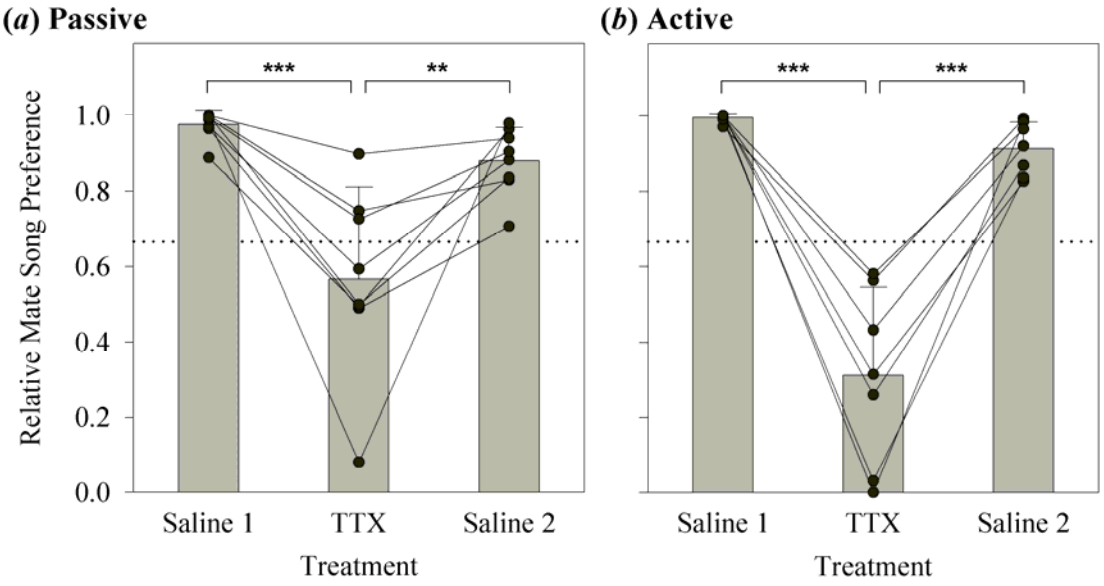

Figure 4.5. Phonotaxis results from (*a*) passive and (*b*) active phases showing relative preferences for the mate's song versus a repertoire-matched novel song [mate/(mate+novel)] following infusions into NCM. (*a*) Mate song preferences were significantly reduced during experimental trials (0.57±0.25, $p < 0.001$) and eliminated in 5 out of 8 birds but were universally restored during Saline 2 trials ($p = 0.001$). (*b*) Mate song preferences were abolished by the experimental treatment in all subjects during the active phase (both $p < 0.001$).

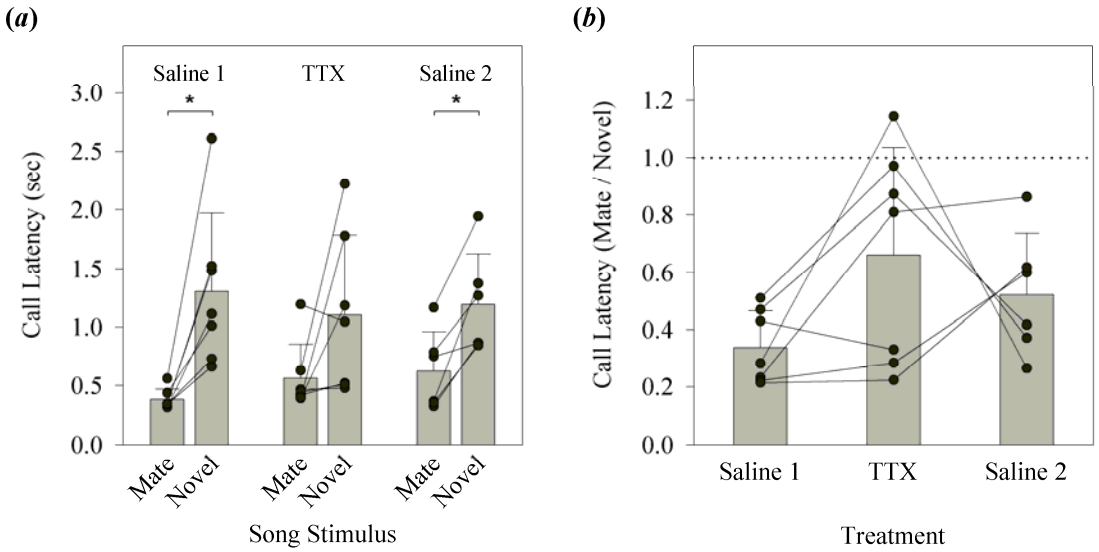

Figure 4.6. Call latency data collected during the passive phase of the mate-novel phonotaxis test following infusions into NCM. (*a*) Subjects called more quickly in response to their mate's song playback than to the novel song in both control trials (both $p < 0.03$) but not in the experimental trials ($p = 0.11$). (*b*) There was no significant difference between treatments when call latencies were expressed as a ratio (mate/novel; $p = 0.10$).

latencies for 3 of the 7 birds were unaffected during experimental trials (Figure 4.6b). There was not a tight correspondence between birds that retained the mate song preference in the passive phase and shorter call latency. Of the five birds that lost their mate song preferences in the phonotaxis task following TTX infusions, three had substantially increased call latency ratios. Conversely, of the three that maintained their mate song preference in the phonotaxis task, one also maintained shorter call latency, one had an increased ratio, and the third was not recorded. No consistent differences in the number of calls between stimuli or across treatments were observed.

Inactivation of NCM also affected female preferences for high quality songs, (Figure 4.7). In the passive phase, this preference (0.89±0.09) was significantly reduced following TTX infusions (0.65±0.27, $p = 0.004$) and restored in Saline 2 trials (0.94±0.07, $p = 0.01$). As before, this reduction remained significant even after removal of the most extreme case (both $p < 0.05$). This effect was not present in the active phase, however. All birds displayed a preference for tutored song in the Saline 1 (0.92±0.05) and Saline 2 (0.80±0.16) trials. This preference was robust to TTX infusions (0.69±0.28, $p = 0.12$), though preferences were abolished in 2 out of 8 birds. As before, there was no side preference effect in either phase (both $p > 0.49$).

(c) *CM inactivation*

In contrast with NCM, inactivation of CM had no effect on mate song preferences (Figure 4.8). In the passive phase, the strong preferences in Saline 1 trials (0.92±0.08) were maintained in TTX (0.86±0.07) and Saline 2 trials (0.89±0.15) ($p = 0.38$). The same was true in the active phase, where mate song preferences in the three trials were 0.89±0.09, 0.78±0.09, and 0.86±0.14, respectively ($p = 0.47$). Notably, TTX infusions into CM did not abolish this preference in any of the subjects during the passive phase and barely did so in one subject during the active phase (preference = 0.65). No side effect preferences were evident (both $p > 0.32$).

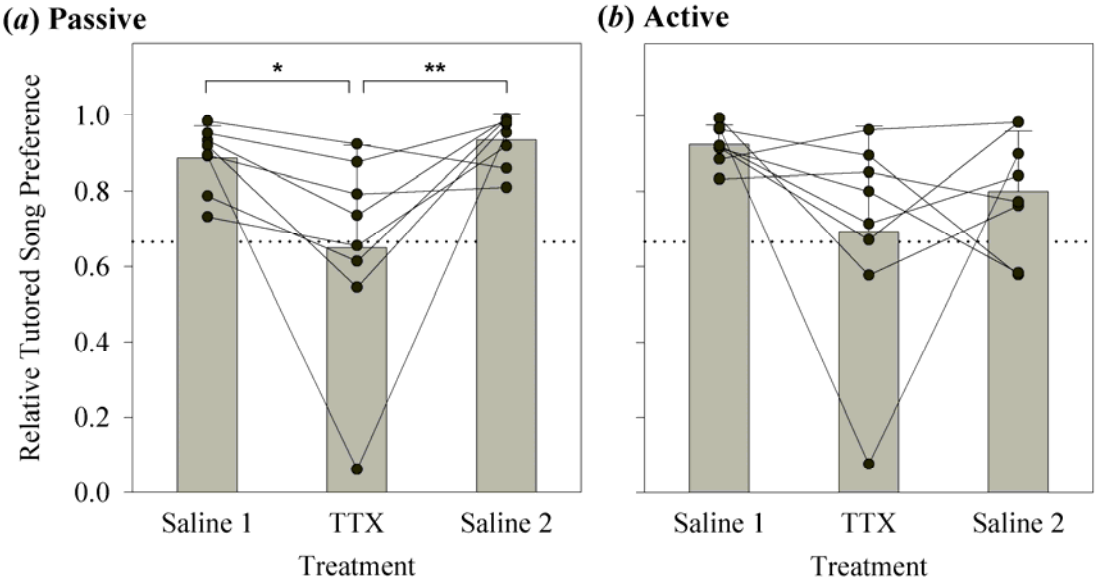

Figure 4.7. Phonotaxis results from (*a*) passive and (*b*) active phases showing relative preferences for a tutored song versus an isolate song [tutored/(tutored+isolate)] following infusions into NCM. (*a*) Tutored song preferences were significantly reduced ($p < 0.05$) following TTX infusions and were eliminated in 4 of 8 subjects, and these preferences were universally restored during the second control trial ($p < 0.01$). (*b*) Tutored song preferences in the active phase were not significantly affected by experimental treatments ($p = 0.12$), though they were eliminated in 2 of 8 subjects.

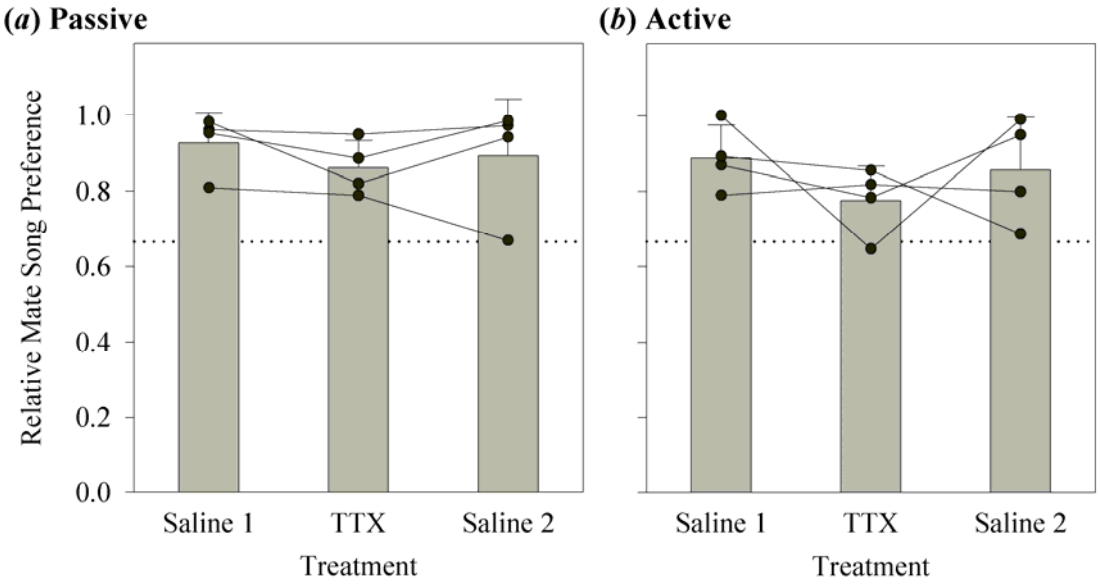

Figure 4.8. Phonotaxis results from (*a*) passive and (*b*) active phases showing relative preferences for the mate's song versus a repertoire-matched novel song [mate/(mate+novel)] following infusions into CM. Experimental treatments did not affect this preference in either phase (both $p \geq 0.38$).

(d) *Cannulae site verification*

Needle tracks within left and right NCM were located 0.42±0.07 and 0.39±0.09 mm from the midline, respectively. All were at least 0.5 mm caudal of the mesopallial lamina (LaM) and stopped approximately half way along the dorsal-ventral axis. Needle tracks within left and right CM were located 0.86±0.11 and 0.91±0.13 mm from the midline, respectively, and were at least 0.3 mm rostral of LaM. Infusions of BODIPY-muscimol were generally contained within NCM or CM and the average drug spread around the needle track was 0.21±0.04 mm^3, though some fluorescence was always detectable along the ventricular borders as well (Figure 4.9).

Discussion

Recent research has delineated pathways within the auditory forebrain of songbirds that are believed to underlie song perception (Vates et al. 1996; Mello et al. 1998). Special attention has been paid to NCM because it is specifically activated by presentation of conspecific song and several lines of evidence suggest that these neurons are involved in song-related memory formation (Mello et al. 1992; Pinaud & Terleph 2008). First, both genomic and physiological responses habituate to repeated presentations of a song stimulus, and these altered responses are specific to individual songs and are long-lasting (Chew et al. 1995, 1996; Mello et al. 1995; Stripling et al. 1997). Second, NCM appears to encode contextual information about specific songs and not simply their acoustic features. Activation levels of the IEG *zenk* are correlated with the strength of learning in associative tasks (Jarvis et al. 1995), and previously learned songs can induce a full *zenk* response when merely associated with a new context (Kruse et al. 2004). Third, both IEG levels (Bolhuis et al. 2000; Terpstra et al. 2004) and physiological habituation rates (Phan et al. 2006) following tutor song playback are correlated with the strength of song learning. Finally, inhibition of *zenk* induction in juveniles disrupts song acquisition (London & Clayton 2008), and lesions

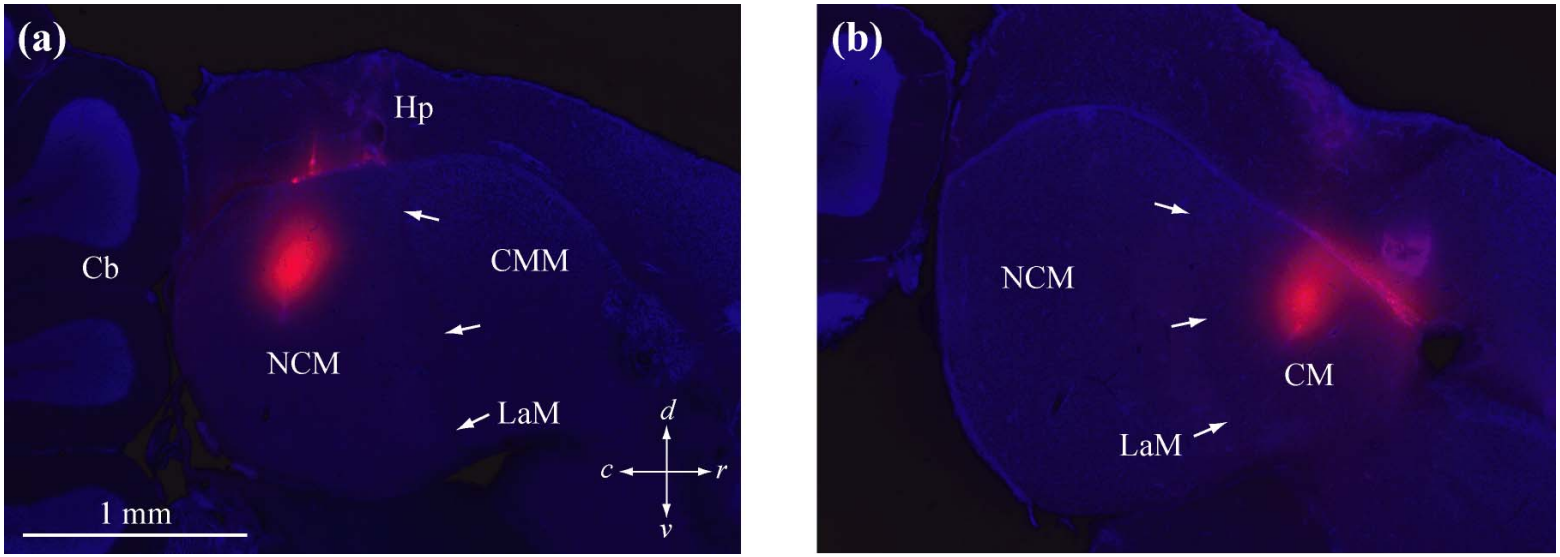

Figure 4.9. Images taken following infusions of 0.15 µl of 1 mM BODIPY-muscimol. Note that the brains were sectioned at an angle to keep NCM intact with the rest of the brain, therefore the relative sizes of other regions are not indicative of needle location along the medial-lateral axis. (a) Needle penetrations into NCM were restricted to the dorsal half and were always ≥0.5 mm caudal of the mesopallial lamina (LaM). The section shown is 360 µm lateral from the most medial section containing NCM tissue. (b) Needle penetrations into CM were generally centrally located and were always ≥0.3 mm rostral of LaM. The section shown is 760 µm lateral from the most medial section containing CM tissue.

of NCM in adults diminish their tutor song preferences (Gobes & Bolhuis 2007). Together, these studies indicate that NCM is important for several aspects of song perception, especially the formation and storage of song-related memories.

Female songbirds provide a useful model system to study the neural mechanisms of song perception because they actively attend to song and exhibit several types of distinct song preferences. This study shows that NCM is necessary for the expression of mate song preferences in adult female zebra finches. This effect does not appear to result from generally lower activity levels or from general perceptual deficits because subjects retained the ability to discriminate species-specific aviary noises from white noise and tones and their preferences for tutored over isolate songs were only modestly affected. These data cannot distinguish between two potential underlying causes, however. One is that the treatment disrupted the neural substrate that encodes song-related memories, in which case the subjects could not retrieve learned songs but their general perception and motivation were unaffected. On the other hand, NCM inactivation may have decreased subjects' fidelity such that they recognized their mate's song but were not compelled to approach that speaker. We attempted to distinguish between these possibilities by analyzing female calls, but the results were ambiguous. Approximately half (3 of 5) of the birds that lost their mate song preference also had comparable call latencies to the two songs, consistent with (but not necessarily indicative of) the idea that these birds did not recognize their mate's song. Yet, two birds that did not prefer their mate's song retained much shorter call latencies, suggesting that they could still discriminate between the stimuli. At least to some extent, then, it appears that decreased pair-bond fidelity contributed to the phonotaxis results. Other potential explanations appear less likely. The reduced mate song preferences do not appear to result from general deficits in song perception or from a general behavioral effect related to the task itself because tutored song

preferences were less affected or even retained. Finally, because inactivation of CM did not affect mate song preferences, this deficit is not attributable to a general brain effect.

In conjunction with previous reports, these results suggest some level of functional compartmentalization within the songbird auditory forebrain. Specifically, NCM appears to underlie the formation and/or storage of song-related memories but not the assessment of song quality. ZENK levels in NCM of female canaries are not related to the proportion of complex syllables within a song (Leitner et al. 2005), nor do levels in female zebra finches differ following playback of directed or undirected song (Woolley & Doupe 2008). They are related to song bout length in European starlings, a feature preferred by females, but only if subjects are first primed with such songs. This may therefore reflect a difference in stimulus salience rather than quality *per se* (Gentner et al. 2001; Sockman et al. 2002). In contrast, neighboring CMM appears to be involved in both functions. Its physiological and IEG activity are elevated in response to familiar songs (Gentner & Margoliash 2003; Gentner et al. 2004; Terpstra et al. 2006), it exhibits greater ZENK activation following higher quality songs (Sockman et al. 2002; Leitner et al. 2005; Woolley & Doupe 2008), and lesions of CMM abolish female preferences for conspecific over heterospecific song (MacDougall-Shackleton et al. 1998). In the present study, inactivation of CM had no effect on mate song preferences, perhaps because the cannulae were directed lateral to CMM in an effort to limit drug spread into Field L or NCM. Infusions centered in more medial regions could perhaps also affect mate song preferences because this subregion is densely interconnected with NCM and its activity reflects song familiarity (Hernandez & MacDougall-Shackleton 2004; Terpstra et al. 2006). Thus, the present results cannot rule out a role for CMM in the expression of preferences for familiar songs.

The neural mechanisms by which NCM may help to encode song-related memories remain unclear. NCM is topographically organized and neuronal tuning bandwidths reflect those of species-typical sounds (Terleph et al. 2006, 2007). Some studies have reported generally lower physiological activity in response to familiar songs (e.g., Phan et al. 2006), but others have not (George et al. 2008). In the latter study, however, NCM neurons were most responsive to the motif class that is most variable between individuals, thus still suggesting an important role in individual recognition. Nevertheless, it remains difficult to speculate about NCM's precise role in the formation of song-related memories because detailed descriptions its intrinsic connectivity and projections are lacking. It currently seems most likely that the projection to CMM, which ultimately projects to multiple auditory and motor areas including the song system (Vates et al. 1996; Bauer et al. 2008), is especially pertinent.

Neurons within the male song system respond selectively to the bird's own song, constituting the most specific auditory response described (McCasland & Konishi 1981). This selectivity emerges gradually through inputs from CM to the interface nucleus of the nidopallium (NIf) to HVC (Janata & Margoliash 1999; Bauer et al. 2008), the last of which gives rise to song-selectivity within other song nuclei (Doupe & Konishi 1991; Vates et al. 1997). With the exception of NIf, this selectivity for single songs has not been observed in the auditory forebrain (Amin et al. 2004), and it also remains unknown if such specificity exists in the song nuclei of non-singing females. Lesions of HVC in female zebra finches do not affect their ability to discriminate conspecific from heterospecific song (MacDougall-Shackleton et al. 1998). In female starlings (which sing), HVC lesions do not diminish their ability to discriminate between familiar songs but they do adversely affect their ability to make novel associations with those songs (Gentner et al. 2000). In female canaries (which

also sing), HVC activity is greater in response to songs with complex syllables (Del Negro et al. 2000) and HVC lesions also affect their discriminatory abilities between songs (Brenowitz 1991; Del Negro et al. 1998; Halle et al. 2002). Thus, the song system can also play an important role in female song perception. It receives highly processed information from the auditory forebrain, and together with it may underlie several forms of song preferences.

This study demonstrates for the first time that NCM is necessary for mate song preferences in adult female zebra finches. NCM likely forms part of the neural substrate that encodes specific song memories and may also underlie aspects of pair bond strength. Future work on its intrinsic circuitry and projections to other systems will help to discover they mechanisms by which song-related memories are formed, stored, and retrieved.

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