The Role of Basal Ganglia-Forebrain Circuitry in the Vocal Learning of Songbirds

by

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Abstract

The basal ganglia form the largest sub-cortical structure in the human brain and are implicated in numerous human diseases. In songbirds, as in mammals, basal ganglia-forebrain circuits are necessary for the learning and production of complex motor behaviors; however, the precise role of this circuitry remains unknown. This thesis is an investigation into how the anterior forebrain pathway (AFP), an avian basal ganglia-forebrain circuit, supports vocal learning in the songbird. This investigation reveals two previously undiscovered functions of the AFP – both related to reinforcement, or trial-and-error, learning.

One necessary component of reinforcement learning is the generation of variable behavior. The songs of learning juvenile birds are naturally highly variable. Rapid pharmacological inactivation of the AFP output nucleus causes an immediate and dramatic reduction in this variability. In addition, the first single-unit recordings of AFP output neurons in singing juvenile birds reveal little correlation with plastic song and a premotor correlation with the most variable form of singing, subsong. These results suggest a novel function for basal ganglia-forebrain circuitry in the production of exploratory behavior.

A second component of reinforcement learning is the evaluation of performance-based feedback – auditory feedback in the case of singing. Conditional disruptive auditory feedback is a novel behavioral paradigm capable of causing rapid experimentally-controlled vocal learning. Inactivating the AFP while using this new paradigm to induce learning reveals that the AFP biases motor output to improve auditory feedback. This result suggests that basal ganglia-forebrain circuits are involved in the evaluation of performance-based feedback. It also suggests for the first time that these circuits are capable of producing temporally precise premotor drive that incrementally improves a motor skill.

In summary, this investigation significantly furthers the view that basal ganglia-forebrain circuitry is involved in reinforcement learning. It ascribes two functions to the anterior forebrain pathway: to drive variable behavior; and to bias future behavior incrementally towards better performance. By analogy, basal ganglia-thalamocortical loops may perform similar functions in mammals.

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Preface

An essay written by my late grandfather and professor of philosophy, Dr. Robert E. Bergmark, seems pertinent to the fulfillment of the academic pursuit that is my thesis. In this essay, he described the philosophies of Democritus and Socrates.

Democritus was a Greek philosopher in the fifth century B.C. His work laid the foundation for all future science and technology. He saw the world as a world of things, not persons; of quantities, not qualities; of mechanistically understood events. People were an outgrowth of these events – matter in motion.

Socrates was a contemporary of Democritus, and he had difficulty accepting this view. His work laid the foundation for all future humanistic belief. He did not believe the qualities and dimensions of human life found in the experiences of love, of commitment, of duty, of trust could be reduced to the world of things. In his view, it was the human experience that infused matter with meaning, purpose, beauty and value.

As both an engineer and a scientist, it is easy to find comfort in the philosophy of Democritus - to embrace the pursuit of understanding the mechanisms of our world as an absolute purpose. But reflecting on the process of completing this dissertation, it is clear to me that this pursuit is secondary.

Without a society concerned foremost with people - their joys and sorrows, their fulfillments and their frustrations, their pleasures and their pains - there can be no pursuit of scientific knowledge. A society that lacks these humanistic concerns is overwhelmed by self-interest and dysfunction.

Thus the principal purpose of education and academia is to foster a society of persons in which personal character is nurtured and supported - for it is the quality of the society that we produce that determines whether the pursuit of knowledge is put to good or evil ends.

With that in mind, I offer my heartfelt thanks to the people who have nurtured me in my pursuit: my thesis advisor and mentor Michale Fee; my remarkable collaborators and co-authors who contributed significantly to this thesis, Dmitriy Aronov and Bence Ölveczky; the members of my laboratory, Timothy Gardner, Jesse Goldberg, Liora Las, Michael Long and Tatsuo Okubo; the technicians in my laboratory, Ricardo Gonzalez and Daniel Rubin; the members of my thesis committee, Markus Meister, Sebastian Seung and Matt Wilson; my advisory committee member Carlos Lois; professors Pawan Sinha and Ted Adelson; my mentor at Xerox PARC, Eric Saund; my mentor at the Carnegie Geophysical Laboratory, Robert Downs; our department administrator Denise Heintze; my colleagues Thomas Davidson and Benjamin B. Scott; my friends Josh Schwab and Randy True; my grandparents Avrum and Anita Andalman and Robert and Carol Bergmark; my parents Martha Bergmark and Elliott Andalman; my brother David Andalman; and my fiancée Wrenn Levenberg.

Chapter 1: Introduction

Understanding the specific function of the basal ganglia in supporting motor learning is important for both basic neuroscience research and clinical medicine. The basal ganglia are found in mammals, birds and reptiles and make up the largest sub-cortical structure in the human brain. Their size and conservation across species, as well as the severe effects that result from disorders of the basal ganglia, suggest they have an essential function in motor learning and control, yet determining that function has proved difficult.

This thesis will address the topic of how basal ganglia-forebrain circuits function to support vocal learning in the songbird. This chapter provides relevant background on four main topics: motor learning, the basal ganglia, vocal learning in the songbird, and the anterior forebrain pathway (the avian homologue of basal ganglia-forebrain circuitry). This chapter closes with an overview of the remaining chapters.

Motor learning

Motor control is a central function of the nervous system

Evidence suggests that brains evolved before bones and that the earliest vertebrates used their brain to decide where to move (Chen, Huang et al. 1999; Mallatt, Chen et al. 2003; Shu, Morris et al. 2003). Thus some biologists believe the name craniates would be preferable to the name vertebrates. In either case, it was likely the ability of the vertebrate brain to coordinate visually guided movement in pursuit of goals, namely food and safety, that provided vertebrates with their primary evolutionary advantage (Carroll 1988; Shadmehr and Wise 2005). In many respects the principal

purpose of the vertebrate brain is the control of movement. The sea squirt makes this point precisely. Their lives consist of two phases – a mobile phase and a sessile phase. During the sessile phase, they literally consume their central nervous system, as it is no longer useful (Llinás 2001).

Motor learning enables adaptive behavior

Evolution has endowed higher vertebrates, particularly humans, with remarkable motor abilities. Importantly, these abilities are not limited to an innate set of evolved reflexes and motor programs. Rather, many vertebrate species have the capacity to both adapt innate motor programs and acquire entirely new motor skills (Doyon and Ungerleider 2003). This motor learning endows organisms with an adaptive and flexible system for motor control that enables success in the face of diverse environments, unpredictable obstacles, and a remarkably complex set of limbs and muscles that require coordination.[1] Demonstrations of motor learning by humans are plentiful and often mesmerizing: think of Michael Jackson's exotic moonwalk, Glenn Gould's fingering at the piano, or Roger Federer's well-placed serve.

Why is motor learning hard?

Operationally defined, motor learning is the process by which movements gradually, through repeated practice, come to be performed effortlessly (Doyon and Ungerleider 2003). The brain faces a daunting problem in attempting to execute this

[1] The term motor learning has both synonyms and alternate meanings. Motor learning can refer to skill acquisition or procedural memory, and is frequently placed within the broader class of non-declarative memory. Additionally the term motor learning has been used to refer to the evolution of motor skills as well as simple types of conditioning like adaptation and sensitization, but these definitions are not applicable to this thesis.

process. The outcome of an action, for instance whether a tennis serve was successful, must be processed by the brain in order to determine which of a billion neurons or 100 billion synapses should be modified such that the timing and coordination of dozens of muscles is better managed in future attempts. Understood in these terms, the brain's ability to succeed at motor learning is undeniably remarkable.

Reinforcement learning: the role of practice in motor learning

Reinforcement learning provides a compelling explanation of how the brain might solve this difficult problem. Reinforcement learning is the formalization of a familiar concept – the concept of trial and error learning (Sutton and Barto 1998). The key insight provided by this formalization is that many extremely difficult problems are solvable provided one can answer a relatively simple question – 'did I do better than usual?'. In other words, the brain does not have to solve the problem of which neurons to make fire in order to serve the tennis ball better. Rather, the brain simply has to ensure that the neurons that fired when it served well, fire again next time. With repetition, or practice, this process yields better performance.

Motor learning is anatomically distinct

Motor learning is biologically distinct from other types of learning and memory. This distinction, in addition to fitting with popular intuition[2], stems from classic work on the severely amnesiac patient H.M. H.M. was treated for epilepsy with bilateral

[2] French philosopher Henri Bergson wrote that habits are "a memory profoundly different... always bent upon action, seated in the present and looking only to the future. ... In truth it no longer represents our past to us, it acts it, and if it still deserves the name of memory, it not because it conserves bygone images, but because it prolongs their useful effect in the present moment" (Bergson, Paul et al. 1912). The quote applies just as well to motor memories.

hippocampal lesions and was rendered unable to recall his activities even a few hours prior (Scoville and Milner 1957). However, scientists Brenda Milner and Sue Corkin discovered that H.M. was still able to improve at motor skills, even over the course of days (Milner 1962; Corkin 1965); H.M was able to learn to draw while only able to observe his hand in a mirror. This finding suggested that while episodic memory (for instance, remembering what one ate for dinner yesterday) involves the hippocampus, motor learning is carried out by a distinct memory system.

The basal ganglia are implicated in motor learning

Basal ganglia anatomy

The basal ganglia are a set of evolutionarily conserved vertebrate brain structures, with homologues found in reptiles, birds, amphibians, and mammals (Marin, Smeets et al. 1998).[3] Across classes, the basic structure of the basal ganglia is conserved; it consists primarily of the striatum and the pallidum. These two regions have different names, locations and anatomy in different species, but the homology is clear. The striatum receives a significant dopaminergic input, and the majority of the neurons in the striatum are GABAergic medium spiny neurons which project to the pallidum (termed the substantial nigra pars reticulata in some species). The pallidum in turn gives rise to a long-range GABAergic projection to the thalamus. Three other pertinent regions are included as part of the basal ganglia because they have reciprocal connections with the striatum and pallidum. These regions are the subthalamic nucleus and dopaminergic cell groups in the substantial nigra par compacta and the ventral tegmental area.

[3] The term basal ganglia was first adopted in the 1950's to describe a set of interconnected sub-cortical brain regions (Packard and Knowlton 2002), but its association with motor control dates as far back as the early twentieth century (Wilson 1912; Wilson 1914).

The most prominent feature of basal ganglia anatomy is the circular loop they form with cortex and thalamus (Fig. 1): the cortex projects to the striatum, which projects to the pallidum, which projects to the thalamus, which projects back to the cortex. These basal ganglia-thalamocortical loops are topographically organized, thus a specific portion of the cortex will connect through the basal ganglia and thalamus and back to itself. In addition, related areas of cortex – for example, somatotopic and motor representations of the hand – can converge on the same area of the striatum (Alexander, DeLong et al. 1986; Flaherty and Graybiel 1993).

Evidence linking the basal ganglia and motor learning

A large body of research suggests the basal ganglia are important for non-declarative learning, of which motor learning is a part. Non-declarative tasks are those in which the learning is expressed in action or performance, rather than words or recollections (Squire 1992; Squire and Kandel 2008). The most convincing studies use the methodology of double dissociation. The experimenter designs two distinct tasks that require similar motivation, perceptual ability and motor skill. This design controls for non-learning related involvement of the basal ganglia. The double dissociation occurs when impairment of the basal ganglia disrupts the ability to learn one task, but not the other; while impairment of another brain region (typically the hippocampus or medial temporal lobe) results in the opposite disruption. The types of tasks for which basal ganglia impairment disrupts learning generally can be categorized as non-declarative (Packard and Knowlton 2002).

The first study to take this well-controlled approach was conducted with rats (Packard, Hirsh et al. 1989). In one task, the win-shift task, the rats used spatial memory to navigate a radial-arm maze task (Olton and Samuelson 1976; Olton and Papas 1979). In the other task, the win-stay task, the rat had to learn to visit radial arms that were illuminated. This task is considered non-declarative because it invokes the stimulus-response habit formation system (Hull 1943). Lesions of the dorsal striatum impaired the ability to master the win-stay task, but not the win-shift task; whereas lesions of the hippocampal memory system had the opposite effect -- thus implicating the hippocampus and basal ganglia in declarative and non-declarative learning, respectively. This finding has replicated in numerous subsequent studies using a variety of tasks (Kirkby, Polgar et al. 1981; Colombo, Davis et al. 1989; Reading, Dunnett et al. 1991; Packard and McGaugh 1992; Adams, Kesner et al. 2001), lesion techniques (McDonald and White 1993; Packard and McGaugh 1996), and species (Fernandez-Ruiz, Wang et al. 2001).

Impairment of learning in humans with diseases of the basal ganglia

A similar dissociation methodology has been employed in human studies, but rather than using lesions, these studies typically compare the task performance of patients with Parkinson's or Huntington's disease[4] to amnesiacs or patients with Alzheimer's disease. These studies largely agree with the animal literature, although as one might expect, the types of tasks that invoke the non-declarative memory system differ in complexity between humans and animals. A simple stimulus response pairing, that takes

[4] Parkinson's and Huntington's are progressive neurodegenerative diseases of the basal ganglia: Parkinson's disease causes death of dopaminergic neurons in the substantia nigra and Huntington's disease causes death of medium spiny neurons in the striatum.

a rat many attempts to learn, is mastered by a human in just a few trials using the declarative memory system. To overcome this difference, tasks designed for humans frequently involve probabilistic relationships. In one such task, known as the weather prediction task (Knowlton, Squire et al. 1994), subjects have to predict the imaginary weather based on which of four cues they are shown at the beginning of the task. Normal subjects performing the task do not report understanding the relationship between the four cues and four weather outcomes, but nevertheless they slowly, after enough repetitions, learn to predict, far better than by chance, the most likely weather based on the cue. Remarkably, amnesiac patients also perform normally on this task, but patients with Parkinson's disease are unable to learn the task (Knowlton, Mangels et al. 1996).

The importance of the basal ganglia in motor learning, specifically, has been shown using the serial reaction-time task (Nissen and Bullemer 1987). In this task key presses are cued one at a time, and unbeknownst to the subject, the key presses are requested in a repeating sequence. Normal controls gradually, with repeated practice, improve their reaction time to the cues, despite reporting being unaware of the sequence. Amnesiacs show the same reaction time improvements (Nissen and Bullemer 1987; Reber and Squire 1994), but patients with Huntington's disease do not show improved reaction times (Willingham and Koroshetz 1993). In addition, imaging studies using PET and fMRI show activation of the striatum during this task in normal non-diseased subjects (Doyon, Owen et al. 1996; Rauch, Whalen et al. 1997).

A reinforcement learning theory of basal ganglia function

The slow and repetition-based forms of learning that require the basal ganglia suggest that the basal ganglia may function as part of a reinforcement learning system. Reinforcement learning boils down to the commonsense idea that if an action results in a better than expected outcome, then the tendency to produce that action should increase. This process slowly progresses toward optimal behavior when two components are present: the exploration of a range of actions and the evaluation of the resulting outcomes. There are numerous studies relating the basal ganglia to the latter of these two components. Functional imaging studies show that blood flow in the striatum is modulated by the amount of reward an action produces (Delgado, Locke et al. 2003; McClure, Berns et al. 2003; Tanaka, Doya et al. 2004); and neurons in the striatum encode for both action value (Samejima, Ueda et al. 2005) and expected reward (Kawagoe, Takikawa et al. 1998). In addition, the large dopaminergic input to the striatum encodes reward prediction error (Schultz and Dickinson 2000), a critical value in reinforcement learning theory (Sutton and Barto 1998). These results, along with others, raise the possibility that the function of the basal ganglia relates to reinforcement learning. But what exactly is this function, and how exactly does it eventually serve to improve motor output? These questions remain unanswered.

Songbirds as a model system for basal ganglia-dependent motor learning

Vocal learning in the songbird has emerged as an important model system in which to study both motor skill acquisition and the function of basal ganglia-forebrain circuitry. Songbirds typically learn to sing by listening to and then subsequently imitating adults of their own species (Thorpe 1958; Thorpe 1961; Immelmann 1969;

Marler 1970). The songbird's capacity to learn and the fact that its singing behavior is complex yet easily quantifiable make it an ideal model system in which to study motor learning[5]. Of the many songbird species, the zebra finch is the most commonly studied species because of its small size, rapid maturation and general robustness (Zann 1996).

The zebra finch song has a hierarchical organization. The song is composed of *syllables*, which are individual sounds separated by silent intervals (Price 1979). The bird produces a set of syllables, each of which has a recognizable spectral structure that is highly conserved between renditions. Multiple syllables are combined into a relatively fixed sequence to form the *motif* of the bird. Finally, motifs are sung in rapid succession, a variable number of times, to form song *bouts* (Sossinka and Bohner 1980).

Songbirds learn their song through a gradual process of trial and error learning

The song learning process in the zebra finch occurs during a critical period early in life (Immelmann 1969; Eales 1985). This critical period begins with a *sensory* phase during which the juvenile zebra finch acquires a memory of a 'template' song which the bird will eventually imitate (Immelmann 1969; Marler 1970; Arnold 1975; Bohner 1983; Kroodsma and Miller 1996). Juvenile birds who are not exposed to song during this sensory period grow up to sing an abnormal song, referred to as isolate song (Thorpe 1958; Marler 1970; Marler and Sherman 1983). Before the sensory phase has ended, the *sensorimotor* phase begins. During this phase, the bird begins to sing. Initially, the spectral structure and ordering of syllables is unstable and highly variable (Immelmann

[5] The field of bird song changed forever when W.H. Thorpe saw the potential of a new machine, the sound spectrograph, (manufactured by Bell Telephone) to better and more accurately analyze the songs of birds. His work revolutionized the field.

1969; Arnold 1975). This stage of singing is called subsong, and is similar to human babbling. Over the next sixty days, the song gradually progresses, with lots of practice, from subsong, through an intermediate stage called plastic song, which is still variable but contains recognizable syllables, to crystallized song (Immelmann 1969). Crystallized song, or adult song, consists of a highly predictable sequence of syllables, each of which is produced with very little spectral variability.

The crystallized song is typically an imitation of the song to which the bird was exposed during the sensory period, usually the song of the father.[6] In this way, the song learning process is goal directed, i.e. driven to a song that is not innately programmed. Successful imitation by the juvenile does not require the presence of the tutor bird or any other bird during the learning process (Tchernichovski, Lints et al. 1999).

The role of auditory feedback in song learning and maintenance

Importantly, auditory feedback during learning is critical to the learning process; the juvenile bird must be able to hear itself sing. Juvenile birds that are deafened after exposure to tutor song, but before crystallization, are unable to successfully imitiate (Konishi 1965; Nottebohm 1968). In fact, auditory feedback remains important in adulthood. Without it, the crystallized song begins to degrade (Leonardo and Konishi 1999), just has human speech degrades following adult deafening (Doupe and Kuhl 1999).

[6] What the juvenile bird eventually imitates can include components from one or more songs they hear during the sensory period (Immelmann 1969; Bohner 1983; Clayton 1987; Williams 1990). This can even include tutoring with a speaker (Adret 1993; Gardner, Naef et al. 2005).

The necessity of auditory feedback for song learning suggests that the juvenile bird is evaluating its own sounds in comparison to the 'template' song in order to produce a better imitation. Songbirds thus offer a model system in which to study the question of how ongoing behavior is evaluated for the purpose of learning – how practice makes perfect.

The song system: the neuroanatomy of singing

Songbirds have a specialized group of brain structures that are devoted to singing behavior – the song system. The song system includes two neural circuits that are critical for singing behavior: the motor pathway and the anterior forebrain pathway (Fig. 2). The motor pathway is where the motor program for adult song production is encoded. It consists of HVC (proper name), which projects to RA (robust nucleus of the arcopallium), the avian homologue of primary motor cortex, which in turn projects to motor neurons in the brainstem (motor nucleus nXIIts) (Nottebohm, Stokes et al. 1976).[7] Lesions to this pathway drastically disrupt adult song production (Nottebohm, Stokes et al. 1976; Simpson and Vicario 1990), and neurons in the pathway exhibit action potentials that are precisely time-locked to the song output (Yu and Margoliash 1996; Chi and Margoliash 2001; Hahnloser, Kozhevnikov et al. 2002; Leonardo and Fee 2005).

[7] The motor neurons of tracheal-syringeal part of the nucleus of the twelfth nerve (nXIIts) project to six pairs of muscles in the syrinx – the songbird equivalent of the vocal chords, or the larynx, in humans (Suthers, Goller et al. 1999). It is coordination of these muscles, along with respiration, that produces the song.

The anterior forebrain pathway is homologous to basal ganglia-thalamocortical loops

The other circuit, the anterior forebrain pathway (AFP), is composed of an interconnected loop of nuclei that are homologous to mammalian basal ganglia-forebrain loops. The circuit is composed of Area X (proper name, a homologue of the basal ganglia with striatal and pallidal neurons intermingled), which projects to DLM (medial nucleus of the dorsolateral thalamus), which projects to LMAN (lateral magnocellular nucleus of the anterior nidopallium, part of the forebrain)(Nottebohm, Stokes et al. 1976; Nottebohm, Kelley et al. 1982), which in turn projects back to Area X(Nixdorf-Bergweiler, Lips et al. 1995; Vates and Nottebohm 1995). The homology between the AFP and basal ganglia-thalamocortical loops is supported by a range of research including comparative development (Marin, Smeets et al. 1998; Reiner, Laverghetta et al. 2004); anatomy and cell morphology (Lewis, Ryan et al. 1981; Soha, Shimizu et al. 1996; Luo and Perkel 1999); electrophysiology (Farries and Perkel 2002); and protein expression (Reiner, Laverghetta et al. 2004). Like mammalian basal ganglia-thalamocortical loops, the projections within the AFP are topographic such that a region of Area X will project through DLM and LMAN back to itself (Johnson, Sablan et al. 1995; Iyengar, Viswanathan et al. 1999; Luo and Perkel 1999). The primary output of the AFP is an excitatory projection from the nucleus LMAN to the motor pathway nucleus RA. It is via this projection that the AFP influences the pathway where the motor program for singing is eventually encoded.

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The AFP is important for song learning

Although the AFP is not required for singing in adult birds, it is necessary for normal song learning. The initial evidence for a role of the AFP in learning came from lesion studies. Lesions to the AFP in adult birds had almost no overt effect on song (Bottjer, Miesner et al. 1984; Nordeen and Nordeen 1993). However, in juvenile birds, lesions of the AFP disrupted the normal learning progression and resulted in poor imitation (Bottjer, Miesner et al. 1984; Sohrabji, Nordeen et al. 1990; Scharff and Nottebohm 1991). In addition, adult song normally degrades following disruption of auditory feedback, but two studies have demonstrated that this degradation does not occur following lesions of the AFP output nucleus LMAN (Williams and Mehta 1999; Brainard and Doupe 2000). These findings suggest a role for the AFP in song learning and, in particular, motor pathway plasticity.

How does the AFP support song learning?

The combination of the AFP's importance in song learning and its homology to basal ganglia-forebrain loops makes it an attractive area for research. The anatomy of the AFP is simple and well delineated compared to the human basal ganglia, which has broad and complex connections with cortex. In addition, the AFP appears specialized for the task of song learning and maintenance, whereas the human basal ganglia support a wide range of behaviors. These attributes make the AFP a strategic and tractable choice for the study of basal ganglia function (Doupe, Perkel et al. 2005).

The loss of variability following LMAN lesions is an important clue

Variable behavior is a critical component of reinforcement learning – without it learning stalls. The song of juvenile birds normally contains significant spectral and sequence variability. Lesions of LMAN in juvenile birds cause a profound reduction of this variability (Scharff and Nottebohm 1991). This change, in many ways, parallels the transition from subsong to crystallized song. Several models explain the AFP's role in learning as being related to this effect.

A prominent theory contends that LMAN provides trophic factors to RA during song learning that maintain the motor pathway in a juvenile, and therefore variable, state. Without these factors, RA becomes adult-like and the motor program becomes more reliably encoded and unable to change. This theory is supported by several lines of indirect evidence. Lesions of LMAN cause cell death in RA (Akutagawa and Konishi 1994; Johnson and Bottjer 1994), but this cell death can be rescued by injection of brain-derived neurotrophic factor (BDNF) (Johnson, Hohmann et al. 1997). Additionally, BDNF has been linked to neurogenesis in the song system which is considered a possible substrate for plasticity (Knowlton, Squire et al. 1994; Rasika, Alvarez-Buylla et al. 1999; Li, Jarvis et al. 2000), and therefore variability. The publication of a comprehensive study showing that lesions of LMAN in juvenile birds have numerous effects on RA (Kittelberger and Mooney 1999) – all increasing RA's resemblance to its adult form – solidified the trophic hypothesis as the most widely held view.

Another theory is that the variability in juvenile song is a consequence of rapid plasticity in the motor pathway, and that lesions of LMAN prevent this plasticity by disrupting the balance of inputs to RA (Scharff and Nottebohm 1991). The projection from HVC to RA is mediated primarily by AMPA receptors, while the projection from LMAN to RA is mediated by a mix of NMDA and AMPA receptors (Mooney 1992; Stark and Perkel 1999). Since NMDA receptors are often critical to mechanisms of synaptic plasticity (Bliss and Lomo 1973; Collingridge, Kehl et al. 1983), perhaps removal of LMAN input destroys the motor pathway's capacity to change, thus eliminating both variability and learning.

A third possibility is that LMAN directly produces variability via excitatory input to RA that is uncorrelated with song timing – i.e. that LMAN provides variable premotor drive. This hypothesis is interesting because it supposes that the motor pathway of a juvenile bird is capable of producing stereotyped song, but that the AFP, a basal ganglia-forebrain circuit, actively introduces exploratory drive. As variable behavior is a key ingredient in reinforcement learning, lesions of LMAN would prevent the song from changing. In this view, variable behavior is not a consequence of immature or noisy neural circuitry (Edelman 1987; Seung 2003), but is the parlance of a dedicated basal-ganglia forebrain circuit.

A new function of the AFP in learning: the production of exploratory behavior

Chapters 2 and 3 present convincing evidence that this third possibility is correct. The AFP indeed drives variability in juvenile song via fast glutamatergic input. These

findings suggest that an important function of basal ganglia circuitry is the production of exploratory behavior – one of the key components of reinforcement learning.

Does the AFP evaluate auditory feedback?

A second key component of reinforcement learning is the evaluation of behavioral outcomes, which is then used to reinforce success. In addition to producing variability, the AFP could participate in this aspect of reinforcement learning: the AFP could evaluate auditory feedback from singing to generate an instructive signal that guides plasticity in the motor program (Brainard and Doupe 2000). Experiments attempting to test this hypothesis have had mixed results. Neurons in the AFP respond both to the bird's own song and the tutor's song outside of singing (Margoliash 1983; Doupe 1997), but these neurons show no change of activity when auditory feedback during singing is manipulated (Hessler and Doupe 1999; Leonardo 2004; Kozhevnikov and Fee 2007).

What form might an instructive signal take?

An AFP signal that guides plasticity in motor pathway could take several forms. One possibility is that the AFP generates a signal which changes the strength of the synapses connecting HVC to RA. These instructed changes would cause the motor program encoded by the motor pathway to better match the template song. An alternative possibility is that the AFP provides a corrective premotor signal to RA, via fast synaptic input, while the bird is singing. This premotor signal would bias the activity in the motor pathway, in real-time, to produce a better imitation. Presumably, AFP-driven premotor bias could eventually be consolidated into the motor pathway for permanent storage.

The AFP biases motor output during learning to improve auditory feedback

Chapter 4 presents compelling evidence that during vocal learning the AFP produces a signal which biases motor output away from vocal errors, suggesting that, indeed, the AFP is involved in the evaluation of auditory feedback. Chapter 4 also presents tantalizing support for the idea that this signal is instructive, i.e. that it guides plasticity in the motor pathway. If basal ganglia-thalamocortical loops serve a similar function in mammals, then it would suggest that they influence behavior by incrementally improving motor performance and subsequently consolidating these improvements to motor cortex.

Summary of Chapters

In summary, the following chapters present a series of experiments which implicate a song-specialized basal ganglia-forebrain circuit, the AFP, in the two key elements of reinforcement learning: exploratory behavior and the evaluation of performance-based feedback.

Chapter 2 presents evidence that the variability found in juvenile birdsong is not merely the result of immature circuitry in the motor pathway. Rather the juvenile motor pathway is capable of producing highly stereotyped adult-like song. Variability is actively added to the motor pathway via excitatory glutamatergic input from the AFP output nucleus LMAN. The experiments include transient reversible inactivation of LMAN and the first awake behaving recordings of individual projection neurons in juvenile LMAN.

Chapter 3 provides further support for the conclusion that vocal variability is produced by the AFP by establishing that the AFP is necessary for the production of subsong – the most variable form of singing, akin to babbling. This chapter shows that single unit activity of neurons projecting from LMAN to RA have a premotor correlation with subsong.

Chapter 4 introduces conditional auditory feedback, a novel method for experimentally controlling learning, and uses this method to establish that during learning the AFP provides corrective premotor signals that improve performance-based feedback. Additionally, the magnitude of this premotor drive is correlated with the amount of plasticity that subsequently occurs in the motor pathway, suggesting an instructive link between AFP signals and plasticity.

Chapter 5 synthesizes these results into a simple method of how the two major neural circuits of the song system interact during vocal learning. The implications of this model are discussed in relation to reinforcement learning and mammalian basal ganglia function. Experiments to both validate and extend this model are also discussed.

Appendix A describes a novel method for manipulation of auditory feedback. This method will allow new experiments aimed at understanding how performance-based feedback is used by the brain.

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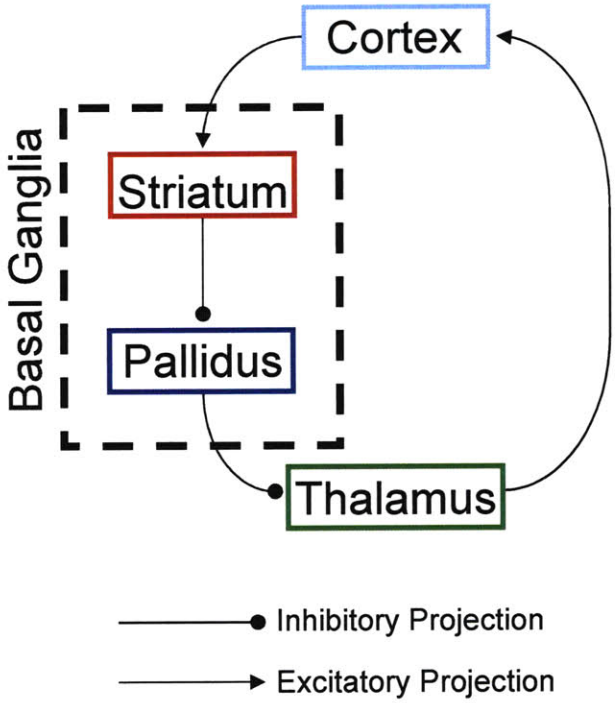
Cortex
Basal Ganglia
Striatum
Pallidus
Thalamus
Inhibitory Projection
Excitatory Projection

Figure 1. The mammalian basal ganglia-thalamocortical loop.

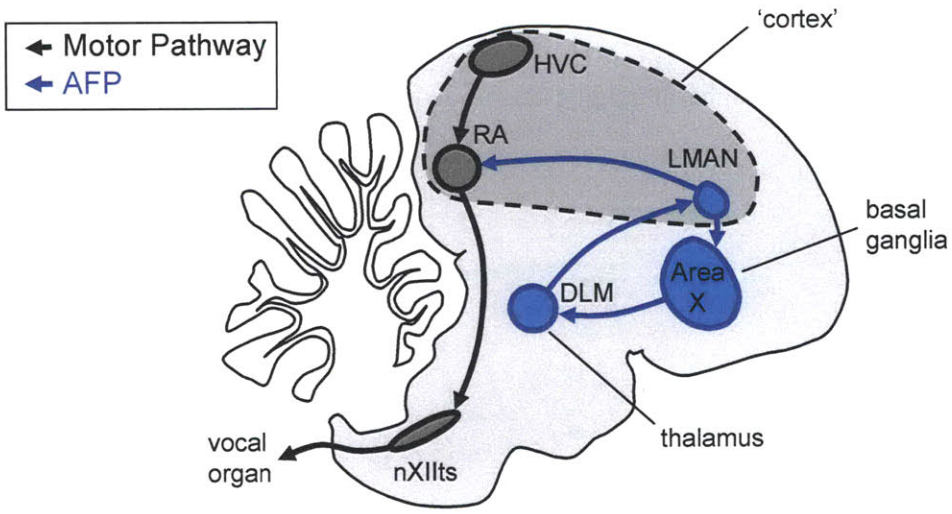
Motor Pathway
AFP
'cortex'
HVC
RA
LMAN
basal
ganglia
Area
X
DLM
thalamus
vocal
organ
nXIIts

Figure 2. The song system. The song system includes two neural pathways: the motor pathway (black) and the anterior forebrain pathway (AFP, blue), a basal ganglia-thalamocortical circuit which consists of Area X (homologue of the basal ganglia, both the striatal and pallidal part), DLM (part of the thalamus), and LMAN (homologue of cortex).

Chapter 2

Vocal experimentation in the juvenile songbird requires a basal ganglia circuit

Attributions.

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A.S.A. contributed to this work by helping develop the method of rapid localized pharmacological manipulation; by developing a lightweight microdrive for recording from juvenile birds; by collecting and analyzing all electrophysiological data; by writing results, methods and discussion pertaining to electrophysiological data; by editing and revising the manuscript.

Abstract

Songbirds learn their songs by trial-and-error experimentation, producing highly variable vocal output as juveniles. By comparing their own sounds to the song of a tutor, young songbirds gradually converge to a stable song that can be a remarkably good copy of the tutor song. Here we show that vocal variability in the learning songbird is induced by a basal ganglia related circuit, the output of which projects to the motor pathway via the lateral magnocellular nucleus of the nidopallium (LMAN). We found that pharmacological inactivation of LMAN dramatically reduced acoustic and sequence variability in the songs of juvenile zebra finches, doing so in a rapid and reversible manner. In addition, recordings from LMAN neurons projecting to the motor pathway revealed highly variable spiking activity across song renditions, showing that LMAN may act as a source of variability. Lastly, pharmacological blockade of synaptic inputs from LMAN to its target premotor area also reduced song variability. Our results establish that, in the juvenile songbird, the exploratory motor behavior required to learn a complex motor sequence is dependent on a dedicated neural circuit homologous to cortico-basal ganglia circuits in mammals.

Introduction

The acquisition of complex motor sequences, such as swinging a golf club or playing the piano, can be thought of as reinforcement learning. This learning process requires the exploration of a range of motor actions and the concomitant evaluation of the resulting performance, reinforcing motor programs that lead to improved outcomes (Sutton and Barto 1998). Similarly, juvenile songbirds explore a large range of

vocalizations by continuously varying their song (Immelmann 1969), utilizing auditory feedback to improve their performance (Konishi 1965). Thus, song learning encompasses the two ingredients of reinforcement learning: exploratory motor behaviour, and performance evaluation.

In the songbird, two main neural pathways are involved in song production and song learning (Fig 1a). The 'motor pathway' controls the vocal motor program through the hierarchical organization of several pre-motor nuclei. A key nucleus in the motor pathway is the robust nucleus of the arcopallium (RA), which projects to brainstem nuclei controlling the vocal and respiratory muscles (Nottebohm, Kelley et al. 1982). During singing, RA neurons in adult birds generate a highly stereotyped sequence of bursts (Yu and Margoliash 1996), which appear to be driven by precisely timed inputs from nucleus HVC (Hahnloser, Kozhevnikov et al. 2002). RA also receives input from the 'anterior forebrain pathway' (AFP), a circuit homologous to the basal ganglia thalamo-cortical loops (Luo, Ding et al. 2001; Farries and Perkel 2002) that may be involved in controlling motor diversity and stereotypy in mammals (Canales and Graybiel 2000). Lesions of the AFP in juvenile zebra finches have devastating effects on song development, whereas the same manipulations in adults have no short-term consequences for song production (Bottjer, Miesner et al. 1984; Scharff and Nottebohm 1991).

While the critical importance of the AFP for song learning has been established, its specific role remains unknown (Margoliash 2002). It has been proposed that the AFP may be involved in comparing the auditory feedback of the bird's vocal output with a stored auditory template of the desired song – an evaluation process that could provide a corrective signal to the motor pathway needed for reinforcement learning(Troyer and

Bottjer 2001). However, recent results showing that the firing patterns of LMAN neurons in adult birds are insensitive to distorted auditory feedback have called this idea into question (Hessler and Doupe 1999; Leonardo 2004). Here we test the alternative hypothesis that, in juvenile songbirds, LMAN is involved in generating vocal variability (Doya and Sejnowski 1995) - the other important ingredient of reinforcement learning.

Results

Our approach was to transiently inactivate LMAN in juvenile zebra finches (n=7 birds, see Methods), and observe whether and how their songs were affected. Birds were briefly head-restrained and injections of a sodium channel blocker, tetrodotoxin (TTX, 30 nL at 50 μM), were made in LMAN in both hemispheres, inactivating the nucleus (see Supplementary Figs 2,3). After injections, birds were returned to a sound-isolated chamber, where they typically began to sing after 0.5-1.5 hours. In all birds probed, LMAN inactivation resulted in an immediate loss of acoustic variability across song renditions. The effect was particularly dramatic in birds at an early stage of song development (~55 days post hatch, dph) because these birds normally exhibit greater song variability. (Fig 1b,c, Supplementary Fig 1, and Supplementary audio).

To quantify song variability, experiments were carried out in slightly older birds with less sequence and acoustic variability (n=6 birds; age range: 59-72 dph). This allowed us to reliably identify song syllables, the basic acoustic units of zebra finch song, across song renditions (Fig 2a). The variability score (V) - a measure reflecting the acoustic variability of a syllable across renditions (see Methods) – was calculated for all identified syllables before and after TTX injection. Without exception, the syllables

showed a highly significant reduction in variability as a consequence of LMAN inactivation (Fig 2b; n=25 syllables; $\langle V \rangle_{\text{before}} = 0.46$, $\langle \Delta V \rangle = 0.2$; $p_{ave} < 0.0001$, t-test). In fact, the juvenile song after inactivation was significantly less variable than songs of adult zebra finches singing undirected song (i.e., songs not directed to a female, Fig 2d, $p < 0.001$; t-test). LMAN inactivation also eliminated 75% of the difference in mean variability between juvenile song and adult directed song – the most highly stereotyped form of song (Kao, Doupe et al. 2005).

To verify that the loss of variability resulted from silencing LMAN neurons, and not from inactivating fibers of passage near LMAN, a $GABA_A$ receptor agonist (muscimol, 25 mM, 30 nL) was injected bilaterally into LMAN (n=2 birds; 66, 70 dph). Again, all syllables showed a dramatic reduction in variability after injection (n=8 syllables; $\langle V \rangle_{\text{before}} = 0.43$, $\langle \Delta V \rangle = 0.16$; $p_{ave} < 0.0001$, t-test). While the reduction in acoustic variability was similar to that resulting from TTX injections (Fig 2b), the duration of the effect of muscimol was substantially shorter than observed for TTX (Fig 2c). This difference in temporal profile was in good agreement with the known in-vivo pharmacology of TTX and muscimol (Martin and Ghez 1999; Boehnke and Rasmusson 2001), suggesting a direct link between suppression of spiking activity in LMAN and loss of variability.

An additional effect of LMAN inactivation was a significant reduction in sequence variability, a measure of the variability in syllable ordering (Fig 2e, $p < 0.005$, paired t-test, see Methods). In fact, the sequential ordering of syllables after TTX injection was comparable in stereotypy to that of adult song. Thus, LMAN activity may influence sequence generation, possibly through an indirect feedback pathway going

from RA to HVC, the putative sequence generator (Schmidt, Ashmore et al. 2004).

We confirmed that the loss of song variability following injections into LMAN did not result from diffusion of the drugs into the medial magnocellular nucleus of the nidopallium (MMAN), a nucleus ~1.25 mm medial from LMAN with projections to HVC. Bilateral injections of TTX into MMAN, done in the same birds in which LMAN injections were previously made, had no significant effect on acoustic variability (Fig 2b).

We next considered the neural mechanisms by which LMAN affects variability in the motor pathway. One intriguing possibility is that song variability is driven by fast synaptic input from LMAN. If true, then acoustic variability should be accompanied by variability in the firing patterns of RA-projecting LMAN neurons. To test this idea explicitly, we recorded single-unit signals from 29 LMAN neurons in singing juvenile birds (n=3 birds; age range: 62-79 dph). In all, 17 of these were antidromically identified as RA-projecting LMAN neurons (see Methods). These neurons exhibited song-related changes in firing rate (12±4 Hz spontaneous, 39±6 Hz during singing, mean±s.d.), and generated significantly more bursts during singing (Fig 3c). Raster plots of the spike trains aligned to the song motif showed that the patterns of spikes and bursts generated by individual neurons were different each time the bird sang (Fig 3a,b).

Correlations in the spike trains across different renditions of the motif were small (0.054±0.34 SD) compared to those observed in premotor neurons of adult birds (0.90±0.1)(Leonardo and Fee 2005). We also compared the correlation distributions to those calculated after random time-shifts were added to the spike trains (see Methods). In general, the distributions of the randomized spike trains were very similar to those

calculated for the motif-aligned spike trains (Fig 3d), confirming that the firing patterns of LMAN neurons are highly variable. Nevertheless, in 13 out of the 17 identified RA-projecting neurons the correlation distributions were still significantly different from those of the randomly shuffled spike trains ($p<0.01$, K-S test), suggesting that while LMAN activity is highly variable, it is not completely random with respect to the song.

Guided by the neural data, we next tested the hypothesis that LMAN drives song variability by providing excitatory glutamatergic input to RA - which in the zebra finch is mediated almost exclusively by N-methyl D-aspartate (NMDA) -type receptors (Mooney and Konishi 1991). In contrast, glutamatergic inputs to RA from HVC are mediated by a mixture of NMDA and AMPA –type receptors (Fig 4a) (Stark and Perkel 1999). Thus if LMAN drives song variability through glutamatergic input to RA, then blocking NMDA receptors should reduce this variability, while sparing the AMPA mediated drive from HVC. In line with our hypothesis, bilateral injections of AP5 (50 nL, 30 mM) into RA significantly reduced acoustic variability in all song syllables examined, (Fig 4b,c; n=4 birds; age range: 57-73 dph; 11 syllables; $\langle V \rangle_{\text{before}} = 0.47$, $\langle \Delta V \rangle = 0.16$; $p_{ave}<0.0001$, t-test). The time course of the variability reduction (Fig 4d) was consistent with the temporal profile of AP5 effects seen in other in-vivo studies (Steele and Morris 1999).

Given that AP5 has effects beyond blocking LMAN input to RA, it may influence the song in ways other than reducing variability. To examine whether AP5 injections affected acoustic structure of syllables, we compared the acoustic features of syllables after AP5 injection to the same syllables before injection (average similarity score 78.0, 11 syllables, see Methods). In comparison, the average similarity score across renditions of the same syllables prior to injection was 77.7, suggesting that the effect of AP5

injection was largely limited to song variability.

Discussion

Previous studies have shown that permanent LMAN lesions in the juvenile bird disrupt song learning and result in an impoverished and prematurely stereotyped song (Bottjer, Miesner et al. 1984; Scharff and Nottebohm 1991). Such lesions are known to produce synaptic maturation in RA within a few days (Kittelberger and Mooney 1999), perhaps due to a loss of neurotrophic input from LMAN (Bottjer, Miesner et al. 1984; Scharff and Nottebohm 1991). Because of the long delay from lesioning to singing (often several days), these studies could not address whether increased stereotypy was caused by synaptic reorganization in RA, or by a more immediate mechanism such as the loss of fast synaptic input from LMAN. In our experiments, we observe singing within an hour after injection, and find that LMAN inactivation reduces song variability reversibly and on a short timescale. This observation implies that, in addition to slow neurotrophic effects, LMAN acts on RA rapidly to drive or control song variability, a necessary ingredient of reinforcement learning. Thus our results suggest that the loss of vocal plasticity following permanent lesions of LMAN may, in part at least, be due to the immediate loss of exploratory behavior.

What is the mechanism by which neural activity in LMAN controls motif-to-motif variability in the song? Our experiments tested the hypothesis that fluctuations in the song are driven directly by synaptic input from LMAN (Stark and Perkel 1999). In this view, the premotor circuit generates a stereotyped song sequence upon which the AFP acts to drive variations. This hypothesis requires that neural activity in LMAN be

highly variable across different song motifs, a prediction that was borne out by our recordings in LMAN (Fig 3). In comparison, premotor neurons in adult birds (singing song of comparable stereotypy to our LMAN-inactivated juvenile birds) generate extremely stereotyped, song-locked spike patterns (Fig 3d) (Yu and Margoliash 1996; Hahnloser, Kozhevnikov et al. 2002; Leonardo and Fee 2005). In itself, the result that LMAN neurons are only weakly time-locked to the song may not be surprising. The significance of this observation becomes apparent when considering that these neurons send excitatory projections to the motor pathway, and that they are necessary for the expression of song variability as demonstrated by our inactivation results. Together with the finding that electrical stimulation of LMAN in adult birds can drive transient changes in the song (Kao, Doupe et al. 2005), these observations make LMAN a likely source for the variability in the premotor pathway.

Because LMAN input to RA neurons is mediated almost exclusively by NMDA receptors, another strong prediction of our hypothesis was that blockade of NMDA receptors in RA should reduce song variability. Our results from the injection of AP5 into RA confirmed this. However, given the presence of NMDA receptors in the projection from HVC to RA (Stark and Perkel 1999), and perhaps in recurrent connections within RA, blockade of NMDA receptors is likely to have effects on RA circuitry other than the loss of direct synaptic input from LMAN. Thus, this experiment cannot preclude other hypotheses — for example, that LMAN acts to regulate stochastic processes intrinsic to the premotor circuit, through some yet unknown mechanism.

Further support for the idea that LMAN directly drives song variability comes from studies in the adult zebra finch. Song-related neural activity in LMAN is variable

also in the adult bird, and this variability has been shown to be larger during undirected as compared to directed singing (Hessler and Doupe 1999; Leonardo 2002). A recent study (Kao, Doupe et al. 2005) linked the increased neural variability in LMAN during undirected singing to an increase in motif-to-motif variability in song features (see also Fig 2d).

How does the role and function of LMAN change as song variability is reduced during learning and finally during song crystallization? To the extent that the variability of LMAN firing patterns in the adult bird during undirected song (Leonardo 2002) is similar to that in the juvenile bird, an essential part of song development may be a reduction of the gain by which LMAN drives RA. This could occur as a result of synaptic changes within RA that weaken input from LMAN and/or strengthen the projections from HVC. While there is evidence that this may indeed occur (Herrmann and Arnold 1991; Kittelberger and Mooney 1999), more experiments are needed to establish how the developmental reduction in song variability is related to changes in song circuitry.

Reinforcement learning requires that variability in the motor output be accompanied by a mechanism that evaluates the resulting performance. In the songbird, such an evaluation signal could be sent directly to the motor system (e.g. to RA), perhaps via a neuromodulator (Schultz 2002), to reinforce the states of the motor pathway that lead to a better-than-expected match to the memorized template. A reinforcement signal could also be sent to the AFP to shape or regulate the fluctuations introduced into the motor pathway via LMAN. This would make LMAN more than a simple "noise generator", allowing it to bias vocal fluctuations in the direction of the desired song. Such bias is suggested by the presence of small but significant correlations in the motif-aligned

firing pattern of LMAN neurons (Fig 3). This bias could permit a more efficient exploration of motor space, and even allow LMAN activity to drive plastic changes in the motor circuitry.

The exploratory motor behavior exhibited by juvenile songbird may also provide general insights into how the brain generates fluctuations required for learning. Such fluctuations could be generated within the motor pathway or by brain regions projecting to it, and could result from stochastic processes, such as randomness in synaptic release (Seung 2003), noise propagated by summation of irregular patterns of IPSPs and EPSPs (Shadlen and Newsome 1998), or from complex collective dynamics of the neuronal network (Kenet, Bibitchkov et al. 2003). Our results strongly suggest that, whatever the detailed biophysical mechanisms, the neural circuits generating these fluctuations are located outside the motor pathway in a specialized pathway involving the basal-ganglia. The output of this circuit acts on the motor pathway allowing the song system to explore the vocal space in a purposeful manner. Whether inducing exploratory motor behavior is a general feature of basal-ganglia circuits is an intriguing idea that remains to be explored.

Methods

Subjects

Subjects were juvenile male zebra finches (54-79 days post hatch). Birds were obtained from the MIT zebra finch breeding facility, and from the aviary at the Rockefeller Field Research Station (Millbrook, NY). The care and experimental manipulation of the animals were carried out in accordance with guidelines of the

National Institutes of Health and were reviewed and approved by the MIT Institutional Animal Care and Use Committee.

Reversible inactivation

Birds underwent a brief surgery to attach to the skull a means of restraining the head during drug injections. The animals were anesthetized with isoflurane (2%) and placed in a stereotaxic apparatus (MyNeurolab.com). Two stainless-steel screws (#0-80 0.25" long) were secured to the skull with dental acrylic. Small holes (~300 μm dia) were drilled through the cranium bilaterally over LMAN, or MMAN using stereotaxic coordinates. The holes were covered with a thin layer of Kwik-Kast (WPI, Inc.). The animals were then placed in a custom sound-isolation chamber where they began to sing prolifically after a few days – typically 200-1000 song motifs per hour.

Inactivation of song control nuclei in the singing bird was carried out by placing the bird, unanaesthetized, in a small foam restraint and attaching the head-mounted screws to a metal plate bolted to the stereotaxic apparatus. The Kwik-Kast over the cranial holes was removed and a 30nL of tetrodotoxin (TTX, 50μM, Sigma #T5651) or Muscimol (25 mM, Sigma #M1523)) was injected bilaterally into the brain region of interest using a Nanoject II injector (Drummond Scientific). The procedure of injecting the birds took ~10 minutes. Experimental confirmation of the physiological effects of TTX injections showed that LMAN was almost completely inactivated after our injections (see Supplementary Figure 3). Regions immediately surrounding LMAN were also affected, and we can not rule out an indirect contribution from the partial inactivation of these regions. For inactivation of NMDA-mediated synapses in RA, 2-

amino-5-phosphonovalerate (AP5, Sigma #A5282) was injected bilaterally into RA (50nL, 30 mM).

Injected solutions also contained dye-conjugated dextrans (Molecular Probes #D22912). All injection sites were verified by histological examination and were found to be within the target nucleus (see Supplementary Figure 2), except for TTX injections in LMAN in two birds: one in which the LMAN injection site in one hemisphere was found to be ~100 μm anterior to the edge of LMAN, the other in which the injections were ~200 μm posterior to LMAN, but right in the middle of the fiber tract leading from LMAN to RA. The results from these birds were similar to those from other birds, and were included in the analysis.

Chronic neural recordings in LMAN

Birds were selected to be at an age in which they produced readily identifiable syllable sequences, yet showed variable acoustic syllable structure across song renditions. Recordings were carried out using a motorized microdrive described previously (Fee and Leonardo 2001). Cells were isolated by searching for spontaneous or antidromically-evoked spiking activity; units typically had signal-to-noise ratios greater than 10:1. Antidromic identification of RA-projecting LMAN neurons was carried out with a bipolar stimulating electrode implanted in RA using techniques described previously for antidromic identification of RA-projecting HVC neurons (Hahnloser, Kozhevnikov et al. 2002). Neurons exhibiting a short-latency antidromic spike (<5 ms) with a RMS latency jitter of less than 100μs (at a stimulation current of ~10% above threshold) were counted as identified RA-projecting neurons. Of the 17 antidromically-identified neurons in our

dataset, 10 were further validated with collision tests (Hahnloser, Kozhevnikov et al. 2002). An additional 10 putative projection neurons did not respond to RA stimulation with a short-latency spike, but exhibited spike patterns and correlations similar to the identified projection neurons. For the cells in our data set, we recorded signals for many song motifs (range 5-133 motifs, mean of 56).

Data Analysis

Song Variability: To assess the effects of drug injections on acoustic variability and average acoustic structure, analysis was done on reliably identifiable song syllables (range: 2-5 per bird, see Fig 2a for an example). Each data point was derived from 45 pairwise comparisons made across 10 consecutive renditions of a given syllable, recorded immediately before and after injection. Acoustic variability was quantified using the Sound Analysis Pro 1.04 software (Tchernichovski, Nottebohm et al. 2000), and pairwise comparisons of the acoustic features of identified syllables were made using the local similarity measure ('accuracy'). This measure is based on pitch, frequency modulation, amplitude modulation, Wiener entropy, and goodness of pitch, and is calculated in 9 ms intervals and averaged over the duration of the syllable; syllables were aligned in time such as to maximize the similarity, allowing for 5 % time warping. For the variability measurements the resulting similarity score (S, ranging from 0-100) was converted, through a linear remapping, to a variability score (V) by the following formula: $V = \frac{S_{\max} - \langle S \rangle}{S_{\max} - \langle S_{\min} \rangle}$. $\langle S_{\min} \rangle$ is the average similarity score of randomly chosen pairs of syllables from unrelated birds, which in our finch colony was measured to be 50±12 (s.d., n=200 pairwise comparisons; comparisons were made across syllables of birds from

different fathers). The similarity of identical syllables, S_{max}, is 100 by definition of the similarity measure. Thus, a variability score of 1 means that syllables are as different as two unrelated syllables, while variability score of 0 means that the syllables are identical. Error bars for V in the figures all denote standard error of the mean. $\langle V \rangle$ denotes the average variability score across birds and syllables for a given condition.

The variability of syllable ordering in a song was quantified using the stereotypy score of Scharff and Nottebohm (Scharff and Nottebohm 1991), excluding the variability in the number of introductory notes and in the end syllable of a song bout. The score is a combination of *sequence linearity*, which addresses the way in which notes are ordered, and *sequence consistency,* a measure of the frequency with which the main motif sequence appears. Complete stereotypy yields a score of 1, while a completely random sequencing will have a score close to 0. Stereotypy scores were calculated over 10 consecutive song bouts, before and after LMAN injections.

LMAN Firing Patterns: The sequence of song syllables most frequently produced by each bird was determined, and motifs that matched this sequence were identified and time-aligned using the onset of one of the syllables. The alignment syllable was chosen for a sharp onset in acoustic power. The relative jitter in the timing of other syllables in the motif was found to be less than 9 ms RMS. Spike times were extracted and the instantaneous firing rate during each motif rendition was estimated by smoothing the spike train with a Gaussian of half-width 20 ms (to the 1/e points). Correlations were calculated between the firing rate functions for all pairs of smoothed spike trains. Correlations were also calculated for all pairs of spike trains after a random time-shift.

The shift was circular such that spikes wrapped around to the beginning of the motif; time shifts were chosen randomly from a uniform distribution with the width of the motif. For each cell the correlation distribution of the time-shifted firing rates was calculated with 100 different ensembles of random shifts. This random shift ensured zero mean correlation while preserving spike statistics. Thus the distribution of time-shifted correlations provides a zero-correlation baseline with which to compare our results.

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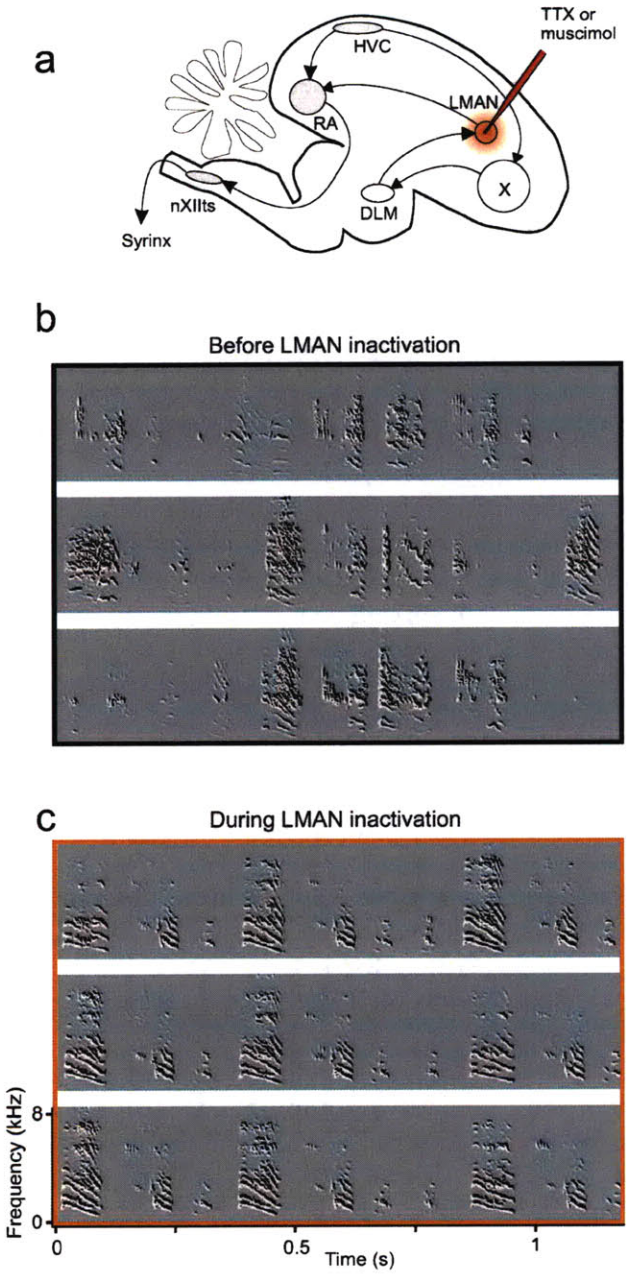
a
TTX or
muscimol
HVC
LMAN
RA
X
nXIIts
DLM
Syrinx
b
Before LMAN inactivation
c
During LMAN inactivation
Frequency (kHz)
8
0
0
0.5
1
Time (s)

Figure 1. Inactivation of LMAN significantly reduces vocal experimentation, making the otherwise variable song of the juvenile zebra finch highly stereotyped. a) Two major pathways in the vocal control system of the songbird. The motor pathway (gray) includes motor cortex analogues HVC and RA, while the anterior forebrain pathway, (AFP, hollow), a basal ganglia thalamo-cortical circuit, consists of Area X, the dorsolateral anterior thalamic nucleus (DLM), and LMAN, which, in turn, projects to RA. To inactivate the output of the AFP, injections of TTX and muscimol (red bolus) were made into LMAN. b) Examples of a juvenile zebra finch song (57 dph) showing large variability in sequence and the acoustic structure of song syllables. c) Inactivating LMAN with TTX produces an immediate reduction of sequence and acoustic variability, revealing a highly stereotyped song produced by the motor pathway. The song snippets shown in (b) and (c) are from consecutive song bouts, immediately before and 1 hour after drug injection. Songs are displayed as spectral derivatives calculated as described (Tchernichovski, Nottebohm et al. 2000). The frequency range displayed is 0-8.6 kHz. For audio of song bouts before and during LMAN inactivation in this bird, refer to Supplementary Audio.

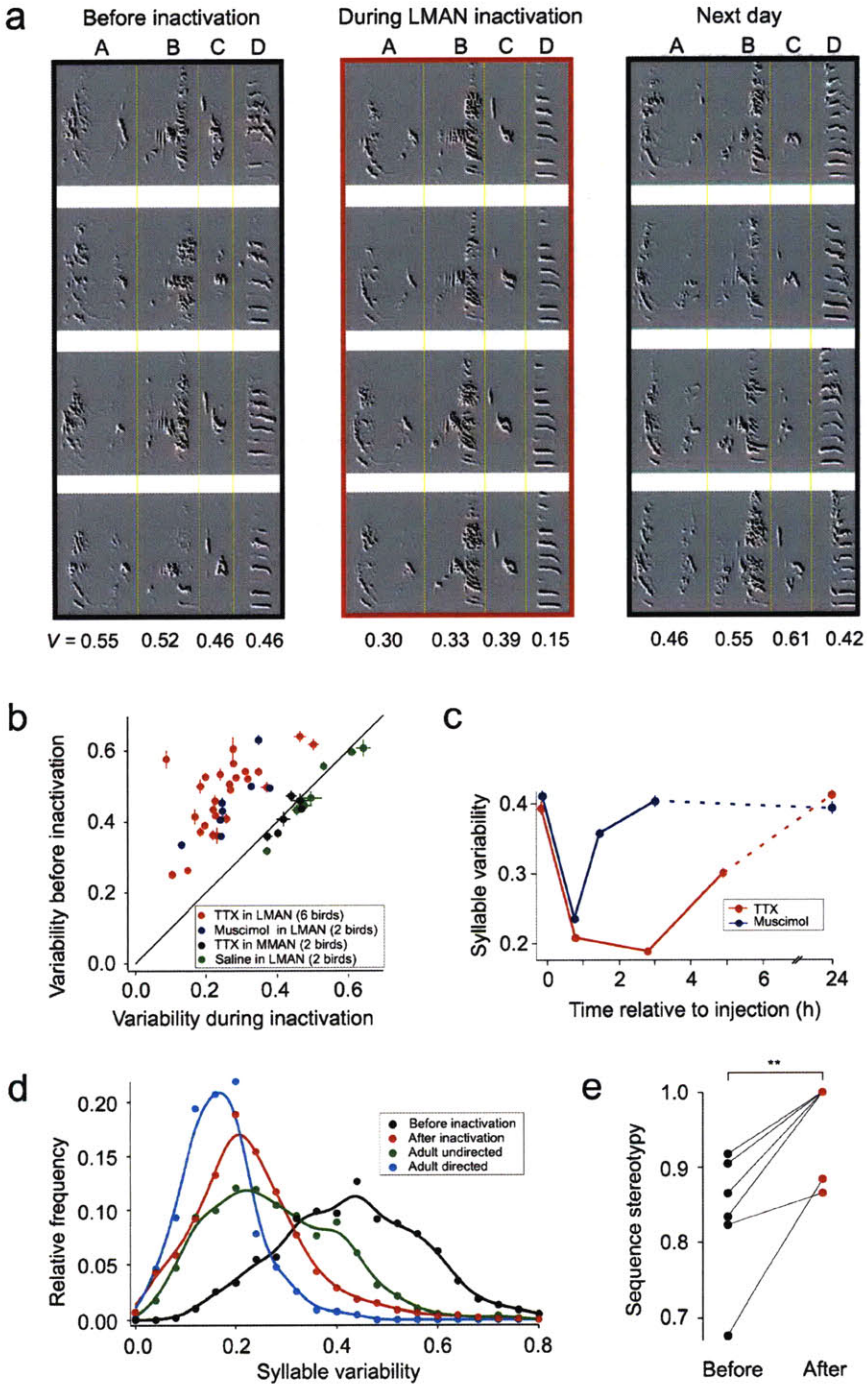
a
Before inactivation
During LMAN inactivation
Next day
A B C D
V = 0.55 0.52 0.46 0.46
0.30 0.33 0.39 0.15
0.46 0.55 0.61 0.42
b
Variability before inactivation
Variability during inactivation
TTX in LMAN (6 birds)
Muscimol in LMAN (2 birds)
TTX in MMAN (2 birds)
Saline in LMAN (2 birds)
c
Syllable variability
Time relative to injection (h)
TTX
Muscimol
d
Relative frequency
Syllable variability
Before inactivation
After inactivation
Adult undirected
Adult directed
e
Sequence stereotypy
**
Before
After

Figure 2. Analysis of the effect of bilateral LMAN inactivation on song variability. a) Consecutive renditions of a repeating song motif of 0.5 s duration in a juvenile bird (59 dph) arranged vertically. Note the large variations in acoustic structure within individual syllables before LMAN inactivation (left panel). Following TTX injection into LMAN, the acoustic variability is dramatically reduced (middle panel), only to return to the original level by the following day (right panel). Numbers below each column indicate the variability index calculated for the 4 renditions of the syllables shown. b) Scatter plot of variability scores before and during LMAN inactivation with TTX (red markers) and muscimol (blue markers). Also shown are results for bilateral TTX injection into MMAN (black markers, see text), and saline injection into LMAN (green markers). c) Time course of variability reduction following TTX (red curve) and muscimol (blue curve) injections show a time dependence that reflects the known in-vivo pharmacology of the respective agents. Data were averaged over four identified syllables and taken from the same bird over consecutive days (dph = 70, 71; muscimol inactivation followed by TTX inactivation). d) Distribution of variability scores for all syllables analyzed in the TTX and muscimol experiments (25 unique syllables, 6 birds) before (black trace) and during (red trace) LMAN inactivation in juvenile birds. Shown for comparison are the variability scores for adult zebra finch syllables (18 syllables, 4 birds; undirected song, green trace; directed song, light blue trace). Markers represent raw data, while the lines are smoothed running averages. e) TTX inactivation of LMAN significantly increased syllable sequence stereotypy. Sequence stereotypy scores (see Methods) for 6 birds before and after TTX injections into LMAN. For comparison, the average stereotypy score for adult birds singing directed song was 0.95 (n=4 birds).

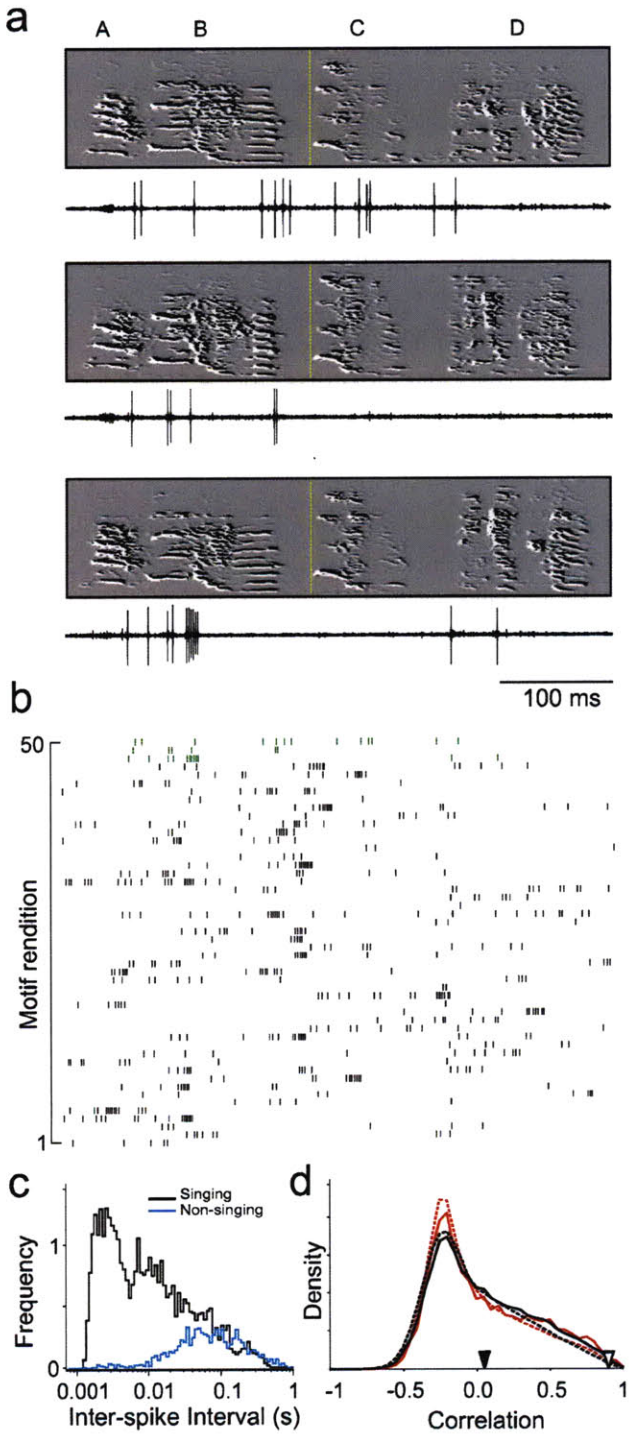
a
A
B
C
D
100 ms
b
50
Motif rendition
1
c
Singing
Non-singing
Frequency
1
0
0.001
0.01
0.1
1
Inter-spike Interval (s)
d
Density
-1
-0.5
0.0
0.5
1
Correlation

Figure 3. Song-aligned firing patterns of RA-projecting LMAN neurons in singing juvenile zebra finches are highly variable. **a)** Three successive renditions of a 67 day old bird's song motif. Displayed under each spectrogram is the simultaneously recorded voltage waveform of an antidromically-identified RA-projecting LMAN neuron (verified by collision testing). Average syllable variability for the three motifs is 0.31. Motif alignment was done at the onset (yellow lines) of syllable 'C'. **b)** Raster plot showing the spike patterns for 50 consecutive motif renditions for the same cell as in (a). The motifs in (a) are in green. **c)** Relative frequency of inter-spike intervals during singing and non-singing for all the 17 identified projection neurons (units of intervals/sec, bin size is 0.04 log units). **d)** Distribution of spike-train correlations across all pairs of motifs for the cell in (b) (solid red line). Correlations calculated with random time-shifts added to the spike trains have a similar distribution (dashed red line, see Methods). Also shown is the correlation distribution for the population of identified projection neurons (solid black line, mean correlation indicated by solid arrow), and for the population with random time-shifts added (dashed black line). In comparison, spike trains of neurons in premotor nucleus RA of the adult bird are highly stereotyped ((Mooney and Konishi 1991) - mean correlation indicated by the hollow arrow).

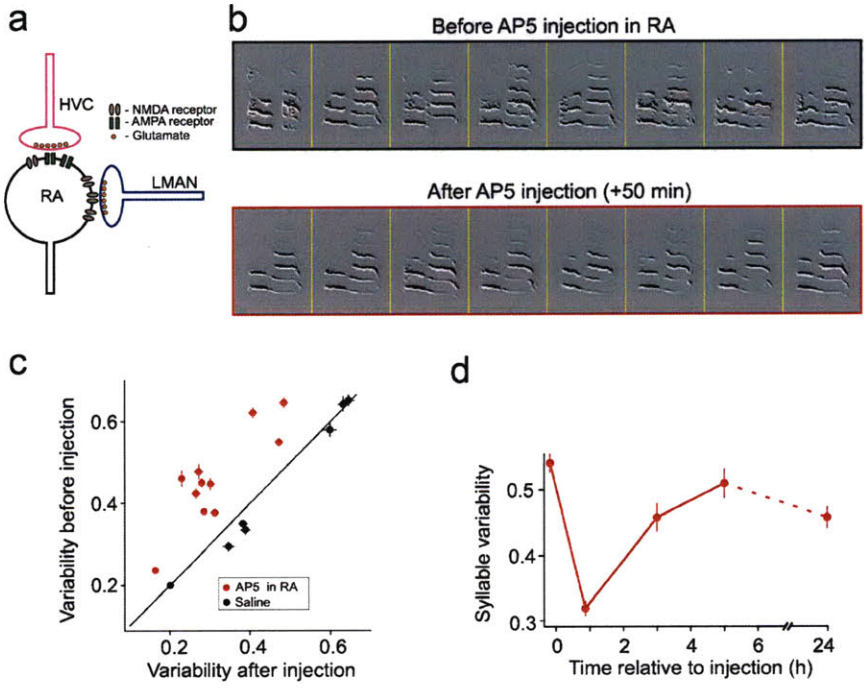
a
HVC
- NMDA receptor
- AMPA receptor
- Glutamate
RA
LMAN
b
Before AP5 injection in RA
After AP5 injection (+50 min)
c
Variability before injection
0.6
0.4
0.2
AP5 in RA
Saline
0.2
0.4
0.6
Variability after injection
d
Syllable variability
0.5
0.4
0.3
0
2
4
6
24
Time relative to injection (h)

Figure 4. Bilateral injections of the NMDA receptor antagonist AP5 into RA significantly reduced song variability. a) Excitatory synaptic inputs to RA from LMAN and HVC are mediated by a different mix of glutamate receptor types (see text). Using AP5 we could block LMAN input while only partially inactivating HVC input. b) Eight sequential renditions of one song syllable in a juvenile zebra finch before and after AP5 injection (63 dph). Note the rapid fluctuations in pitch, the appearance of noisy acoustic structure, and variations in syllable duration before injection. The average variability scores (V) before and after injections for the 8 shown syllable renditions were 0.50 and 0.25 respectively. c) Following injection of AP5 into RA, fluctuations in acoustic structure were substantially reduced. Variability scores of 11 syllables in 4 birds before and after injection of AP5 into RA. d) Time course of acoustic variability following drug injection averaged over all identifiable syllables for the bird in (b).

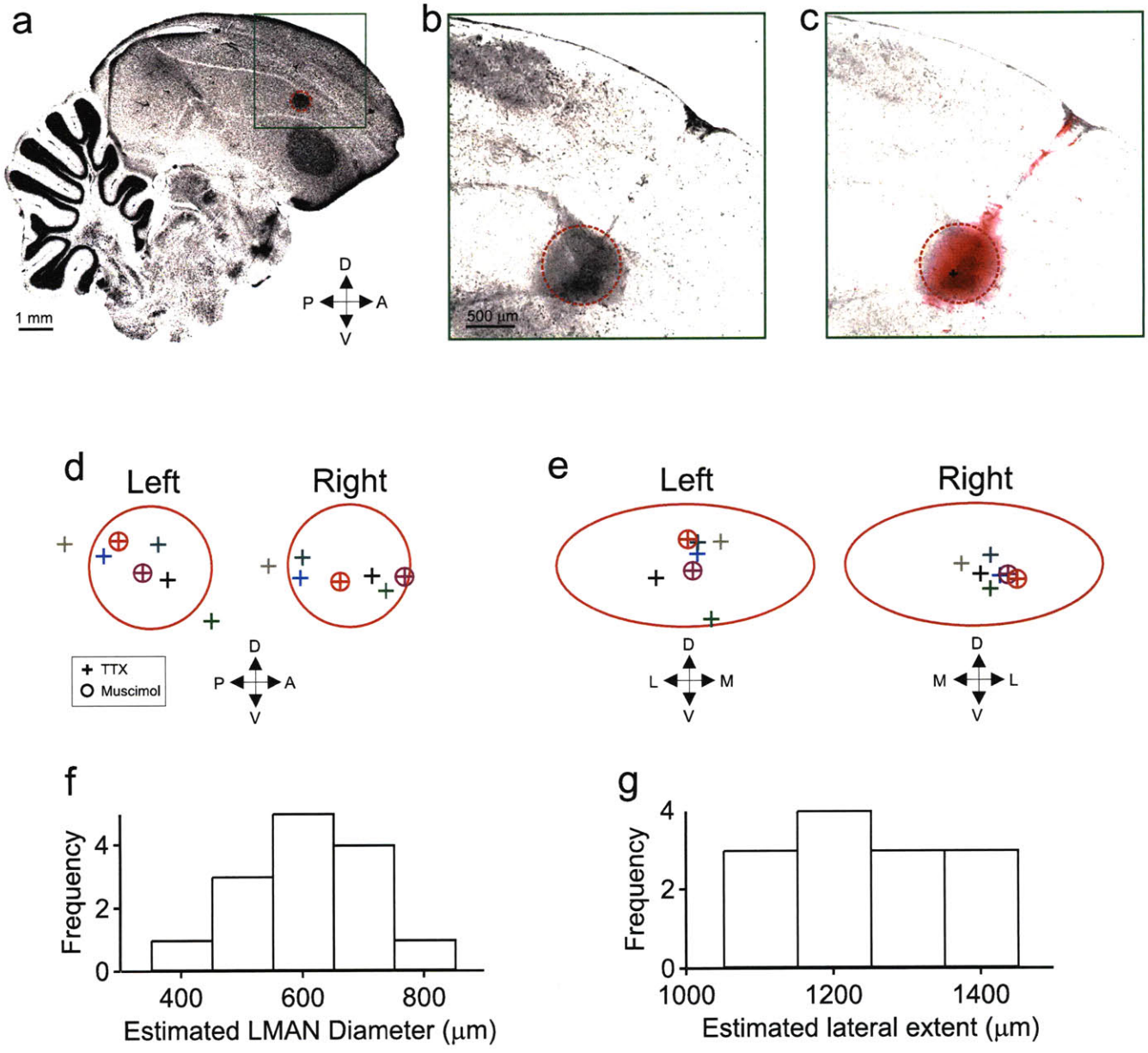
a
b
c
1 mm
500 µm
D
P
A
V
d
Left
Right
TTX
Muscimol
e
Left
Right
M
L
f
Frequency
Estimated LMAN Diameter (µm)
g
Frequency
Estimated lateral extent (µm)

Figure S1. Histology Confirming the Injection Sites for the LMAN Inactivation Experiments in Figures 1 and 2. (A) A parasaggital Nissl-stained section of a zebra finch brain showing the location of LMAN. (B) Inverted darkfield image showing LMAN in one of the juveniles injected (red markers in [D] and [E]). (C) Combined darkfield and fluorescence image showing the spread of the dye that was co-injected with the drug. (D and E) Estimated injection sites relative to the boundaries of LMAN for all birds in Figures 1 and 2 in the saggital (D) and coronal (E) planes, respectively (individual birds are color coded). (F) Estimated maximum diameter of LMAN in the saggital plane. (G) Estimated lateral extent of LMAN in the coronal plane. The estimates in (F) and (G) are based on the contrast borders seen in the darkfield images (see [B]). Note that fibers from LMAN to RA leave the posterior edge of LMAN.

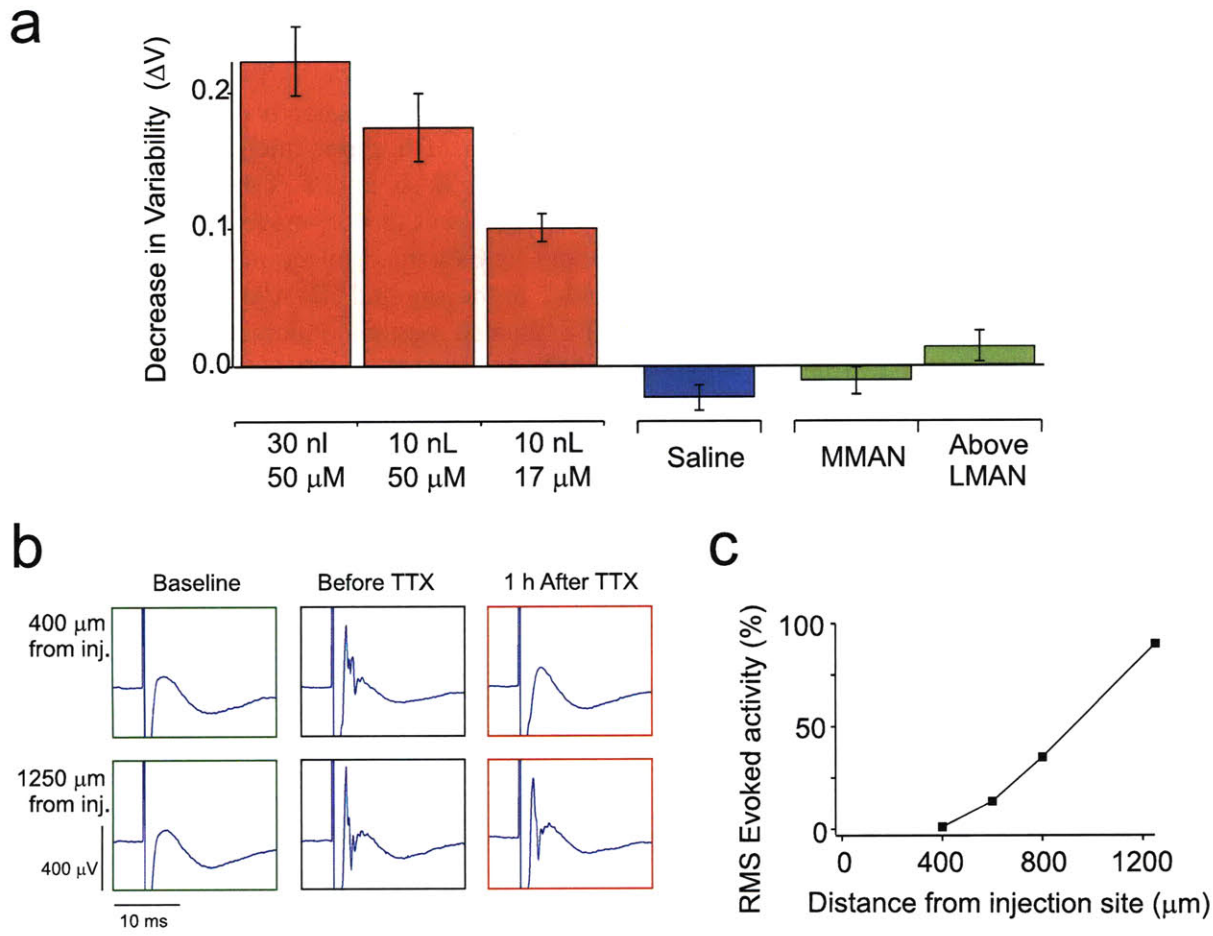
a
Decrease in Variability (ΔV)
0.2
0.1
0.0
30 nl
50 μM
10 nL
50 μM
10 nL
17 μM
Saline
MMAN
Above
LMAN
b
Baseline
Before TTX
1 h After TTX
400 μm
from inj.
1250 μm
from inj.
400 μV
10 ms
c
RMS Evoked activity (%)
100
50
0
0
400
800
1200
Distance from injection site (μm)

Figure S2. Dose- and Distance-Dependent Effects of TTX Injections in and around LMAN. (A) Decrease in acoustic variability (ΔV) approximately 1 h after injection, as a function of location and concentration of TTX injections. Red bars indicate dose response for TTX injections in LMAN (n = 2 birds; 8 syllables; injection sites for the two birds correspond to the blue and grey markers in Figure S1). Blue bars indicate 30-nl saline injections in LMAN (n = 2 birds; 7 syllables). Green bars indicate 30-nl (50 μM) TTX injections 1.25 mm medial (MMAN, n = 2 birds; 6 syllables) and dorsal ("above," n = 2; 8 syllables) from the center of LMAN. (B and C) Summary of experiments done to verify the physiological spread of TTX. Experiments were done in anesthetized birds (2% isoflurane). A bipolar stimulating electrode was placed in RA, and a recording electrode in LMAN, producing antidromically evoked activity in LMAN (stimulus pulses, 175 μA, 0.2 ms, 0.5 Hz). TTX (30 nl, 50 μM) was injected at different distances away from the recording electrode. (B) Examples of recorded signals for TTX injections 400 μm (top) and 1,250 μm (bottom) away from the recording electrode (averaged over 30 stimulus pulses). The baseline stimulus artifact recorded 1 mm above LMAN is shown in the green boxes (left). Signal recorded in LMAN immediately before injection is shown in the black boxes (middle). Signal recorded 1 h after injection is shown in the red boxes (right). (C) Summary of evoked activity 1 h after TTX injections made at different distances away from the recording site. Evoked activity was measured as the root-mean-squared deviation of the signal from the baseline in the interval 1.5–4.5 ms after the stimulation pulse (six birds, two at 400 μm, two at 600 μm, and one each at 800 μm and 1,250 μm).

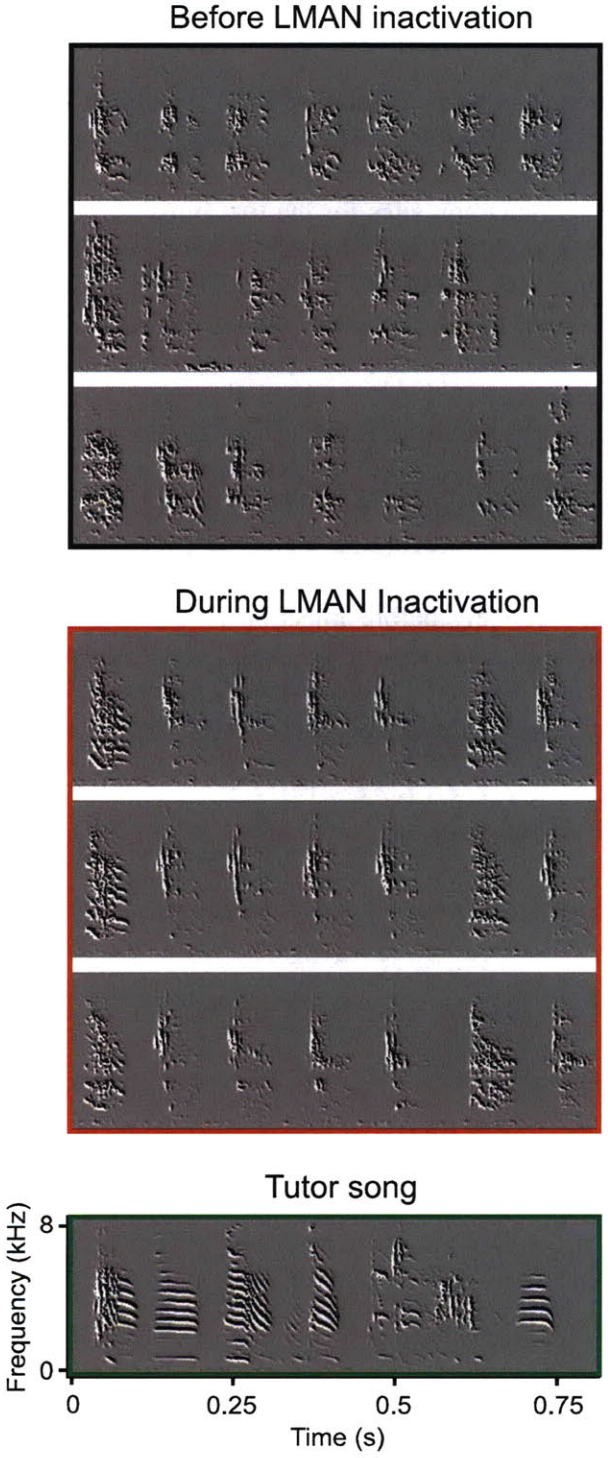
Before LMAN inactivation
During LMAN Inactivation
Tutor song
Frequency (kHz)
8
0
0
0.25
0.5
0.75
Time (s)

Figure S3. Example of a Juvenile Zebra Finch Song (54 dph) Showing a Loss of Sequence and Acoustic Variability following LMAN Inactivation by TTX Injection. The song snippets shown are from three consecutive song bouts, immediately before and 1 h after TTX injection. Tutor song is shown for comparison.

Chapter 3

A specialized forebrain circuit for vocal babbling in the juvenile songbird

Attributions.

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A.S.A. contributed to this work by developing the method of localized pharmacological manipulation using passive reverse microdialysis; by helping to use this method to inactivate HVC, which provided that initial evidence that adult zebra finches produce subsong-like sounds without HVC activity; by helping in the design and construction of 3-D printed microdrives for recording from subsong birds; by collecting electrophysiological data from RA-projecting LMAN neurons in subsong birds; by providing guidance and software for the analysis of the data, in particular the electrophysiological data; by editing and revising the manuscript.

Abstract

Young animals engage in variable exploratory behaviors essential for the development of neural circuitry and adult motor control, yet the neural basis of these behaviors is largely unknown. Juvenile songbirds produce subsong – a succession of primitive vocalizations akin to human babbling. We found that subsong production in zebra finches does not require HVC (high vocal center), a key premotor area for singing in adult birds, but does require LMAN (lateral magnocellular nucleus of the nidopallium), a forebrain nucleus involved in learning but not in adult singing. During babbling, neurons in LMAN exhibited premotor correlations to vocal output on a fast time scale. Thus, juvenile singing is driven by a circuit distinct from that which produces the adult behavior – a separation possibly general to other developing motor systems.

Introduction

How does a young brain learn to use the muscles it controls and the sensory organs by which it perceives the world? To a surprising extent, this knowledge is not built in by deterministic developmental rules but must be obtained through exploration. For instance, the relationship between feedback from the somatosensory periphery and movement is revealed to the developing brain by spontaneous muscle twitches, which facilitate the self-organization of spinal reflex circuits (Petersson, Waldenstrom et al. 2003) and cortical somatosensory maps (Khazipov, Sirota et al. 2004; Milh, Kaminska et al. 2007). At a higher level, juvenile animals learn the causal relation between actions and the effects of these actions by producing highly variable behaviors such as infant stepping, grasp-like "hand babbling," early vocalizations, and play (Fagen 1981; Doupe

and Kuhl 1999; Robinson, Blumberg et al. 2000; Wallace and Whishaw 2003; Imada, Zhang et al. 2006).

How are these exploratory juvenile behaviors generated? Are they produced by the same brain areas responsible for the corresponding adult behaviors later in life, or are specialized brain regions involved? Forebrain areas, including the motor cortex and the basal ganglia, have been implicated in the production of normal infant movements, as well as their abnormalities (Prechtl, Einspieler et al. 1997; Forssberg 1999; Eyre, Miller et al. 2000; Robinson, Blumberg et al. 2000). Yet the specific forebrain circuits for infant motor control remain to be identified.

Babbling is an early motor behavior produced by juveniles of vocal mammals and birds (Marler 1970; Reiss and McCowan 1993; Elowson, Snowdon et al. 1998; Doupe and Kuhl 1999; Knornschild, Behr et al. 2006). In zebra finches, babbling, called subsong, occurs roughly from ages 30 to 45 days post-hatch (dph). Plastic song follows, with the gradual appearance of distinct and identifiable, but variable, vocal elements (syllables). By 80 to 90 dph, plastic song is gradually transformed into highly complex, stereotyped motifs – sequences of syllables that constitute adult song. The premotor circuit for adult song production consists of HVC (high vocal center), RA (robust nucleus of the arcopallium), and brainstem motor nuclei (Fig. 1A). This "motor pathway" is crucial for generating stereotyped, learned vocalizations (Nottebohm, Stokes et al. 1976; Simpson and Vicario 1990) and exhibits firing that is precisely time-locked to the song output (Yu and Margoliash 1996; Chi and Margoliash 2001; Hahnloser, Kozhevnikov et al. 2002; Leonardo and Fee 2005).

Another circuit, the anterior forebrain pathway (AFP), is homologous to basal ganglia thalamocortical loops in mammals and projects to RA through a forebrain nucleus, LMAN (lateral magnocellular nucleus of the nidopallium) (Bottjer, Halsema et al. 1989; Farries and Perkel 2002). Although LMAN is not required for singing in adult birds, it is necessary for normal song learning in juveniles (Bottjer, Miesner et al. 1984; Scharff and Nottebohm 1991) and plays a role in producing song variability in adult and juvenile birds (Kao, Doupe et al. 2005; Ölveczky, Andalman et al. 2005). These and other studies have suggested a view that the motor pathway drives singing, whereas the output of the AFP modulates or instructs the motor pathway during learning (Doya and Sejnowski 1995; Troyer and Bottjer 2001).

Subsong persists in the absence of HVC

We investigated whether primitive subsong vocalizations result from an immature form of the adult motor pathway, or whether they are driven by other premotor circuits. Given the importance of HVC for mature singing (Nottebohm, Stokes et al. 1976; Hahnloser, Kozhevnikov et al. 2002; Thompson and Johnson 2007), we sought to characterize its involvement early in development. In nine subsong-producing juvenile birds (ages 33 to 44 dph), we eliminated HVC bilaterally, either by electrolytic lesions or by pharmacological inactivation (see supplementary information). In three additional birds, we left HVC intact but specifically eliminated its projection to RA by bilateral transection of the HVC-to-RA fiber tract. After these manipulations, all birds continued producing largely unaffected subsong (Fig. 1A and fig. S3).

Surprisingly, older birds – those in the plastic-song stage (45 to 73 dph, $n = 12$) and adults ($n = 5$, undirected singing) – also sang after bilateral HVC elimination (but see supplementary information). These birds lost structure and stereotypy in their songs, reverting to the production of subsong-like vocalizations. After pharmacological inactivation of HVC, this reversion to subsong-like vocalizations was fast (within 20 min) and reversible (fig. S4); this finding suggested that the effect is not due to long-term changes in neural circuitry, but rather occurs immediately as a result of the loss of spiking activity in HVC. At all ages, singing in the absence of HVC was produced at normal rates and followed an ordinary circadian rhythm, with more songs produced in the morning than in later parts of the day (see supplementary information).

Singing without HVC is highly similar to normal subsong

We asked whether the sounds produced in the absence of HVC were indeed similar to subsong. We characterized acoustic properties of songs by measuring spectral features shown to be effective for quantifying developmental trends in zebra finches (Tchernichovski, Nottebohm et al. 2000; Deregnaucourt, Mitra et al. 2005). Distributions of these features before and after HVC elimination were highly similar for subsong-producing birds (see supplementary information). An additional feature of normal subsong is the absence of repeatable acoustic elements of a stereotyped length. This was evident in a wide, unimodal distribution of syllable durations for subsong-producing birds ($n = 9$ birds younger than 45 dph; Fig. 2, A and B). After HVC elimination, these distributions were unchanged (see supplementary information). In contrast, plastic and adult songs contain distinct syllables that form multiple narrow peaks in the distributions of durations. After HVC elimination in older birds, all distinct syllables were lost,

resulting in uni-modal distributions similar to those of subsong (n = 25 birds) (see supplementary information).

Furthermore, subsong is characterized by a lack of sequential stereotypy, which appears later in plastic and adult songs. We quantified stereotypy by measuring the peak of the spectral cross-correlation between different song renditions (Fig. 2C) (see supplementary information). In control conditions, stereotypy was higher for older birds (Fig. 2D; $P < 0.0001$ for nonzero slope of the linear regression of stereotypy and age). However, independently of age, stereotypy was reduced to the level of sub-song after HVC elimination (Wilcoxon $P > 0.1$ for the difference from normal subsong). In summary, analyses of acoustic structure indicate that, by a wide range of measurements, singing in the absence of HVC is highly similar to normal subsong.

Subsong requires activity in RA and LMAN

If subsong persists in the absence of HVC, what neural circuits are engaged in its production? One possibility is that subsong does not require the forebrain song system and is entirely produced by midbrain or brainstem circuitry, even in the absence of RA. A second possibility is that subsong is driven by circuitry intrinsic to RA, even in the absence of HVC and LMAN. The third possibility is that subsong is driven by, or requires, inputs from LMAN to RA. We tested these hypotheses by lesions and inactivations of RA and LMAN.

RA lesions entirely blocked singing in juvenile birds (n = 5, 39 to 73 dph), indicating that subsong-like vocalizations require descending inputs from the forebrain (Fig. 3). Similarly, song production was abolished by lesions of HVC and subsequent

inactivation of LMAN (n = 12 experiments in 5 birds, 51 to 75 dph), indicating that RA circuitry, without its afferent inputs, is not sufficient to generate singing. We further tested the necessity of LMAN inputs to RA by inactivating LMAN in juvenile birds. LMAN inactivation entirely abolished subsong production in all birds younger than 45 dph (n = 6 experiments in 4 birds). However, in agreement with previous studies, LMAN inactivation did not block singing in most older birds (6 of 7 experiments in 5 birds, 45 to 67 dph), although it produced a marked reduction in song variability (Kao, Doupe et al. 2005; Ölveczky, Andalman et al. 2005). Together, these results indicate that RA and its inputs from LMAN are necessary for subsong production.

LMAN neurons exhibit premotor activity during subsong

An intriguing possibility suggested by the above results is that LMAN drives subsong production – i.e., that it generates patterns of spiking activity that control the acoustic structure of subsong on a short (10 ms) time scale. To test this prediction directly, we recorded from single RA-projecting LMAN neurons during subsong production in intact birds [n = 15 neurons in 3 birds, 38 to 45 dph (*31*)] and in birds with bilateral HVC lesions (n = 16 neurons in 2 birds, lesioned at 38 and 50 dph). To quantify premotor activity, we examined firing in a short window preceding each syllable boundary (onset or offset). To begin with, we only considered syllable boundaries separated from other onsets or offsets by relatively long (>150 ms) periods to eliminate the possible confounding effects of neighboring syllables on the firing pattern. There was a significant increase in firing before syllable onsets in 12 of 31 neurons [16.1 ± 1.6 Hz in a 50-ms window preceding syllable onset versus 8.6 ± 0.6 Hz in a 100-ms baseline period preceding this window; $P < 0.05$; e.g., neuron 3, Fig. 4, A and B (*see*

supplementary information)]. Similarly, syllable offsets were preceded by a significant increase in firing in 5 of 31 neurons (21.2 ± 3.4 Hz before syllable offset versus baseline, 15.5 ± 1.3 Hz; $P < 0.05$; e.g., neuron 14, Fig. 4, C and D). Similar neuronal firing patterns related to onsets and offsets of behavioral sequences have been observed in other basal ganglia–related circuits (Fujii and Graybiel 2003).

In the above analysis, we only considered syllable boundaries separated by long (>150 ms) periods of time to isolate syllable onset- and offset-related changes in firing. However, the firing of some LMAN neurons also correlated with more rapid changes in song structure. For instance, neuron 12 (Fig. 4, E to G) exhibited increased firing before syllables that followed short (10 to 150 ms) rather than long intervals, as well as a reduction in firing during silent periods between syllables. Overall, seven neurons showed a premotor increase in activity before syllables separated by short intervals ($P <0.05$ for the comparison of a 30-ms window preceding a syllable with 30 ms of baseline). This finding suggests that some LMAN neurons may have a premotor relation to subsong structure at the level of individual syllables.

In neurons that exhibited a significant increase in firing before syllable onsets (n = 18), high-frequency bursts of spikes (>100 Hz) preceded 13.2 ± 1.4% of syllables. The most likely timing of a burst onset was 17.2 ± 3.1 ms before syllable onset. Such latency is, in fact, anticipated for premotor activity in LMAN, given the 10- to 15-ms latency reported for vocal perturbation after electrical stimulation in RA (Fee, Kozhevnikov et al. 2004) and the 2- to 5-ms antidromic latency from RA we found in LMAN neurons (see supplementary information). Note that although the exact relationship of firing to song varied across cells, 20 of 31 neurons we recorded (65%) showed some type of

premotor correlation to the vocal output. Premotor firing in LMAN did not require activity within HVC; 8 of 16 neurons exhibited significant correlations to song structure in HVC-lesioned birds (fig. S5).

Discussion

Our data indicate that LMAN, and possibly other components of the AFP, constitute an essential premotor circuit for the production of early babbling. At the same time, we have shown that the classical premotor nucleus HVC (Nottebohm, Stokes et al. 1976) is not necessary for the generation of subsong. We therefore propose that two premotor pathways in the songbird function to produce vocalizations at different stages of development. In young juveniles, the AFP generates poorly structured subsong, whereas in adult birds, the classical HVC-motor pathway generates highly stereotyped motor sequences. These pathways interact in the intermediate plastic-song stage (Ölveczky, Andalman et al. 2005) to generate the partially structured but variable vocalizations upon which vocal learning operates.

The transfer of functional dominance from one pathway to another during vocal learning elegantly parallels their anatomical development. HVC does not reach its adult size until the late plastic-song stage (Alvarez-Buylla, Ling et al. 1992) and establishes functional synapses in RA later than LMAN does (Mooney 1992; Mooney and Rao 1994). Song maturation and the decrease in vocal variability have thus been attributed to the strengthening of inputs from HVC and the concurrent weakening of inputs from LMAN (Herrmann and Arnold 1991; Akutagawa and Konishi 1994; Kittelberger and Mooney 1999; Stark and Perkel 1999). Curiously, although HVC neurons form

synapses in RA around the onset of singing [30 to 35 dph (Mooney 1992)], our results show that they do not significantly contribute to song production in its earliest stage. It is therefore possible that the HVC-to-RA pathway is active during early subsong but is not yet functionally strong enough to drive singing by itself or to influence vocalizations in a detectable way.

Identifying forebrain circuits involved in the production of juvenile behaviors is a requisite step toward understanding the mechanisms by which sensorimotor learning takes place. Several models of developmental learning suggest that early motor behaviors originate in the same circuits that later produce adult behavior. In this view, known as neuronal group selection theory, an initially large number of motor patterns undergo a selection process through competition, gradually eliminating circuits that produce undesirable behaviors (Edelman 1987; Sporns and Edelman 1993; Marler 1997; Forssberg 1999; Hadders-Algra 2000). Our findings, however, suggest a rather different model in which distinct specialized circuits are dedicated to the generation of highly variable juvenile behavior. We speculate that similar circuits for the production of infant behavior may be a general feature of developmental learning in the vertebrate brain.

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MATERIALS AND METHODS

Sound recordings

Subjects were juvenile and adult male zebra finches of various ages (>30 dph). Birds were obtained from the Massachusetts Institute of Technology breeding facility. Animal care and experiments were carried out in accordance with the National Institute of Health guidelines and approved by the local Institutional Animal Care and Use Committee. Birds were placed in custom-made sound isolation chambers and vocalizations were recorded either with Sound Analysis Pro (Tchernichovski, Nottebohm et al. 2000) or custom-written software. Thresholds used for triggering sound recordings were substantially lower than those commonly used (1000 ms "minimum peak record duration" with a minimum of 5 "peaks crossing the threshold" in Sound Analysis Pro) in order to ensure recording of all quiet subsong vocalizations. Our settings were sufficient for capturing sounds as quiet as "tet" calls and feather ruffles. To estimate frequencies of songs and calls, we segmented an entire day of recordings into 1-sec segments. We then estimated the numbers of these segments containing calls and songs by manually browsing through a random subsample of 1000 of these segments and directly counting the numbers of vocalizations. In 4 adult birds, we also recorded directed vocalizations by presenting a female bird in a separate cage (see Analysis of Acoustic Features).

Surgery and lesions

Prior to surgery, birds were anesthetized with 1-2% isofluorane in oxygen and placed in a stereotaxic apparatus. Craniotomies were made bilaterally above RA, HVC, or LMAN. RA was identified with a carbon fiber electrode (0.4-0.8 MΩ; Kation Scientific) by the presence of characteristic spontaneous activity. To localize HVC or LMAN, a bipolar stainless steel stimulating electrode was implanted in RA. Current pulses (200 μs at 1 Hz, 50-200 μA) were then delivered with the electrode and HVC or LMAN were localized by the presence of short-latency (2-5 ms) antidromic responses. Lesions in HVC or RA were made with a platinum-iridium electrode (Micro Probe; 100 μA current for 60 s). A 3-dimensional lattice of 6-10 lesions spaced at 250 μm was made in each hemisphere for complete bilateral lesions. Birds were returned to sound isolation chambers, and vocalizations were recorded for at least 10 days following surgery.

In some animals (n=5), a retrograde tracer was injected into RA during the same surgery (20-30 nl of alexa-conjugated dextran or cholera toxin subunit β, Invitrogen). After the experiment, the animal was sacrificed and perfused with 3-4% paraformaldehyde. The brain was extracted and sliced parasagittally for histological examination. We confirmed completeness of HVC lesions by observing the absence of retrogradely-labeled cells in HVC, but the presence of labeling in LMAN (Fig. 1c).

Pharmacological inactivation

For pharmacological inactivation of HVC or LMAN, we devised probes to perform reverse microdialysis without physically restraining the birds (Fig. S4). Probes

consisted of a reservoir (cap of a 23-gage hypodermic needle) connected by a polyimide tube to a concentrically attached 500-μm-long tube of dialysis membrane (Spectra/Por). In this design, a pharmacological agent could diffuse freely from the reservoir down the tube and across the dialysis membrane. The length of the polyimide tube was chosen to reach the region of interest from the brain surface. A smaller polyimide tube was inserted into the dialysis tube and used as flush outlet. All attachment points, as well as the end of the dialysis tube, were sealed with bio-compatible epoxy (Epo-Tek).

Probes were implanted bilaterally into HVC or LMAN and attached to the skull using dental acrylic. In 3 birds, we recorded spontaneous activity in the vicinity of the dialysis probe under anesthesia to calibrate drug concentrations necessary for inactivation of a correctly-sized brain region (Fig. S4). We found that, for complete inactivations of HVC or LMAN, concentrations of muscimol or TTX (Sigma) in the reservoir of the probe needed to be ~500 times higher than those used for direct injections (Ölveczky, Andalman et al. 2005). For inactivation in freely-behaving birds, animals were briefly placed in a small foam restraint and drug (0.016 mg/ml TTX or 1.5 mg/ml muscimol) was applied to the reservoir. For washout, the drug was flushed out of the reservoir and substituted with phosphate-buffered saline (PBS).

Fiber tract transections

We performed bilateral transections of the HVC-to-RA fiber tract by making 3 incisions with an ophthalmic knife (Sharpoint) at fixed stereotaxic coordinates (~500 μm posterior to HVC, between 1.5 and 3.5 mm lateral, 2.5 mm maximum depth). In the same

surgery, we injected a retrograde tracer (see above) into RA, as well as a tracer of a different wavelength into area X (40 nl). In these birds, we confirmed completeness of the transections by observing retrograde labeling of X-projectors, but not RA-projectors in HVC, while observing both tracers in LMAN (Fig. S3).

Electrophysiology

Recordings in LMAN were carried out using a motorized microdrive described previously (Fee and Leonardo 2001). A stimulating electrode was implanted in RA and cells were isolated by searching for spontaneous or antidromically evoked activity. The signal-to-noise ratio was typically 5-15:1. Putative RA-projecting neurons exhibited short-latency (<5 ms) responses to antidromic stimulation with a jitter of less than 100 μs. Of the 31 antidromically identified neurons that were recorded during singing, 27 were further confirmed as RA-projecting by collision tests (Hahnloser, Kozhevnikov et al. 2002). Each neuron was recorded during the singing of multiple subsong bouts (14-681 bouts, average 138).

Data analysis

All data analyses were performed with custom-written software in Matlab and Sound Analysis Pro for Matlab (SAM).

Song analysis

For syllable segmentation in each recording, we calculated a sound threshold as the Fisher discriminant of two Gaussian modes (corresponding to noise and sound) fit to

the values of log-amplitude. We detected crossings of this threshold and defined sound onsets and offsets as the closest points to these crossings where amplitude deviated from noise by 2 standard deviations. Sounds separated by <7 ms of silence were merged into a single syllable, and segments of sound <7 ms long were eliminated. Bout were defined as sequences of syllables separated by at least 500 ms. Song and call rates were quantified on the full day immediately preceding each surgery and on the first full day of singing following each surgery. For experiments that abolished singing, call rates were quantified on the second day after surgery.

To quantify the level of stereotypy, 10 bouts were randomly selected from the data. We only considered bouts that were at least 2 s long, in order to include at least 2 song motifs for adults and late plastic-song birds. For each pair of bouts, a correlation matrix was calculated by computing the correlation of power spectra (between 850 Hz and 8.5 kHz) for each pair of points in time (1-ms spectrogram windows). We then measured the maximum value of the lag correlation function, excluding points within 1 s from either end of the function. The resulting values were averaged across the 45 (10x9/2) comparisons. For regression analysis of stereotypy and age (Fig. 2d), we assigned all adults the age of 90 dph.

Spectral features (see Analysis of Acoustic Features) were measured on each time slice of the spectrogram (1 ms long window). We measured these features on time slices occurring during syllables randomly selected from the data. At least 100,000 time slices were included in the distributions of features (Fig. 3e).

Analysis of neuronal recording

For analysis of neuronal recording, instantaneous firing rates (IFRs) were calculated as inverses of inter-spike intervals. Bursts were defined as events with IFR exceeding 100 Hz. For assessment of premotor activity, average firing rates were quantified in a test window and a baseline window (see text). The ratio of the test rate to the baseline was measured. For each neuron, we asked whether this ratio was significantly above chance level. To evaluate this, we created 1000 surrogate datasets in which syllables and intervals were randomly rearranged within a song bout. The start of the bout was also jittered by a Gaussian-distributed value with standard deviation equal to the average syllable length. P-value was calculated as the fraction of the surrogate datasets for which the ratio of the test firing rate to the baseline was above the ratio for the real dataset.

ANALYSIS OF ACOUSTIC FEATURES

Our results show that zebra finches produce singing highly similar to subsong following bilateral lesions or inactivations of HVC. Here, we quantify these similarities by comparing singing before and after HVC elimination using an array of acoustic features. These features have proven to be effective for quantifying developmental trends in zebra finches and are widely used for quantifying song similarities and differences across experimental conditions (Tchernichovski, Nottebohm et al. 2000; Deregnaucourt, Mitra et al. 2005). Details concerning the calculation of these features have been described previously (Tchernichovski, Nottebohm et al. 2000; Deregnaucourt, Mitra et al. 2005). Here, we provide a brief description of each one, along with the quantification of our data.

In the first section, we focus on the songs of subsong-producing birds (<45 dph) before and after HVC elimination. In the second section, we analyze developmental changes in singing and the effects of HVC elimination on the songs of older birds.

HVC elimination in subsong-producing birds (<45 dph)

Wiener entropy

Wiener entropy measures the width and uniformity of the power spectrum. Broadband noise has high entropy values, whereas pure tones and harmonic sounds have

low entropy values. HVC elimination had no effect on the entropy of subsong. Distributions of this feature before and after HVC elimination were nearly identical (Fig. S1a), with no significant differences in either entropy mean (Wilcoxon p=0.67) or variance (p=0.67).

Pitch goodness

Pitch goodness quantifies the periodicity of the spectrum of a particular sound. Thus, harmonic sounds have high values of pitch goodness, whereas broadband noise and pure tones have low values of pitch goodness. HVC elimination had no effect on the pitch goodness of subsong (Fig. S1b). There were no differences between the means (Wilcoxon p=0.60) or variances (p=0.22) of this measure.

Relative amplitude

Amplitude measures the power of sound across all frequencies. Since power in our recordings is un-scaled, we measured amplitude relative to the baseline noise level in the sound isolation chamber separately for each recording. Amplitude was not affected by HVC elimination (Fig. S1c) and exhibited no significant differences of means (Wilcoxon p=0.14) or variances (p=0.06) between the two conditions.

Frequency modulation

Frequency modulation (FM) is the angular component of squared time and frequency derivatives of sound. Sounds whose frequency is not changing in time have FM values close to 0 degrees. Sounds with rapidly changing frequency have values closer

to 90 degrees. Frequency modulation was not affected by HVC elimination (Fig. S1d) and had no significant differences of means (Wilcoxon p=1) or variances (p=1) between the two conditions.

Pitch

We estimate pitch of sounds using a method adapted for zebra finch songs. At each point in time, we measure harmonic pitch, i.e., the fundamental frequency of sound. Whereas this measure is well-defined for harmonic sounds, it is not appropriate for pure tones or broadband noises. Therefore, for sounds that have high values of Wiener entropy (>-3) or low values of pitch goodness (<100) we consider pitch to be the average frequency of sound (i.e., the center of spectrum gravity in the frequency domain) instead.

Pitch is the only feature that was effected by HVC elimination in subsong-producing birds (Fig.S1d). Following HVC elimination, average pitch was reduced to 85.1+/-2.1% of control across the 9 birds younger than 45 dph (Wilcoxon $p<0.001$). Although this reduction was significant, pitch in the absence of HVC was well within the range of normal subsong. In fact, compared to the control pitch, average pitch following HVC elimination was shifted at most by 0.28 standard deviations (0.18+/-0.03 on average).

Could direct inputs from HVC to RA influence subsong pitch? We analyzed pitch distributions before and after bilateral transections of the HVC-to-RA fiber tract in subsong-producing birds (Fig. S3). Pitch was not effected by these transection (99.5+/-

1.4% of control across 3 birds). Thus, direct inputs to RA via the HVC-motor pathway appear to have no influence on this feature. A possible explanation of this result is that pitch is effected by inputs from HVC to the AFP in subsong-producing birds.

Syllable duration

Distributions of syllable durations are shown in Fig. 2b. In subsong-producing birds, HVC elimination had no effect on syllable duration (113+/-7 ms in control songs, 115+/-10 ms in the absence of HVC, p=0.86). Similarly, there was no effect on the variance of syllable durations (p=0.80).

Bout duration

We defined bouts as sequences of syllables separated by more than 500 ms of silence. HVC elimination had no effect on bout duration (2.57+/-0.21 s in control, 2.13+/-0.22 s in the absence of HVC, p=0.22).

In summary, singing following HVC elimination in birds younger than 45 dph is highly similar to normal subsong by all measures. The only significant difference (reduction in pitch) cannot be explained by disruption of the HVC-to-RA motor pathway. Thus, HVC inputs to RA appear to have no detectable behavioral contribution to the production of subsong.

HVC elimination in plastic-song and adult birds

Following complete bilateral HVC elimination, birds in the plastic-song stage (n=12, 45-73 dph) and adults (n=5) produced subsong-like vocalizations. To our knowledge, this is the first report of singing following bilateral HVC lesions. These songs were produced only in social isolation (undirected singing). When adult birds were presented with a female, a condition that would normally elicit directed singing, lesioned birds did not sing, although they otherwise exhibited normal approach behavior. This result is consistent with earlier studies (Nottebohm, Stokes et al. 1976; Simpson and Vicario 1990) that reported an absence of directed singing following bilateral HVC lesions in the canary and the zebra finch. Regression of song structure similar to that we observe has been reported following partial HVC lesions in the zebra finch (Thompson and Johnson 2007). In addition, left HVC lesions in the canary produced a similar regression (Nottebohm, Stokes et al. 1976). Given the left-hemispheric dominance of singing in the canary, these unilateral lesions may be functionally equivalent to bilateral lesions in the zebra finch.

Singing by plastic-song and adult birds following bilateral HVC elimination was similar to normal subsong. Fig. S2 shows the averages and variances of acoustic features before and after HVC elimination for birds in various age groups. Wiener entropy, pitch goodness, amplitude, and pitch exhibited significant developmental changes ($p<0.05$ for linear regression of means and variances with age across birds, consistent with (Deregnaucourt, Mitra et al. 2005). Following HVC elimination, the means and variances

of these features changed dramatically, in all cases acquiring values more similar to those of normal subsong.

In addition to pitch (see above), two features of plastic and adults songs in the absence of HVC were different from normal subsong: syllable durations and the variance of the entropy distribution.

Syllable durations

Compared to normal subsong, average syllables were shorter following HVC elimination in birds older than 45 dph (85+/-5 ms for plastic-song birds and 79+/-9 ms for adults, compared to 113+/7 ms for subsong; $p<0.05$ in both cases; Fig. 2b). Though shorter on average, syllables produced by these birds were within the range of normal subsong; on the syllable duration distribution of any subsong-producing bird, average durations of all birds without HVC were within 0.6 standard deviations from the mean.

Entropy

Although average entropy values were not affected by HVC elimination, the variances of entropy distributions were lower for plastic-song and adult birds after HVC elimination compared to normal subsong (0.35+/-0.05 in both cases, compared to 0.56+/-0.04 for normal subsong, $p<0.02$). This indicates a greater variation in the quality of sounds produced during normal subsong. Notably, this difference between normal subsong and songs of birds in the absence of HVC is small compared to the

developmental change (factor of 2.43 increase in entropy variance from subsong to adult song).

In summary, singing by plastic-song and adult birds in the absence of HVC is similar by many measures to normal subsong, but exhibits some significant differences. Since we show that LMAN is required for singing in the absence of HVC, these differences could be due to developmental changes in LMAN circuitry or its connections to RA. Alternatively, they could result from changes in any other nuclei upstream or downstream of LMAN, or even from developmental changes in the peripheral vocal organ.

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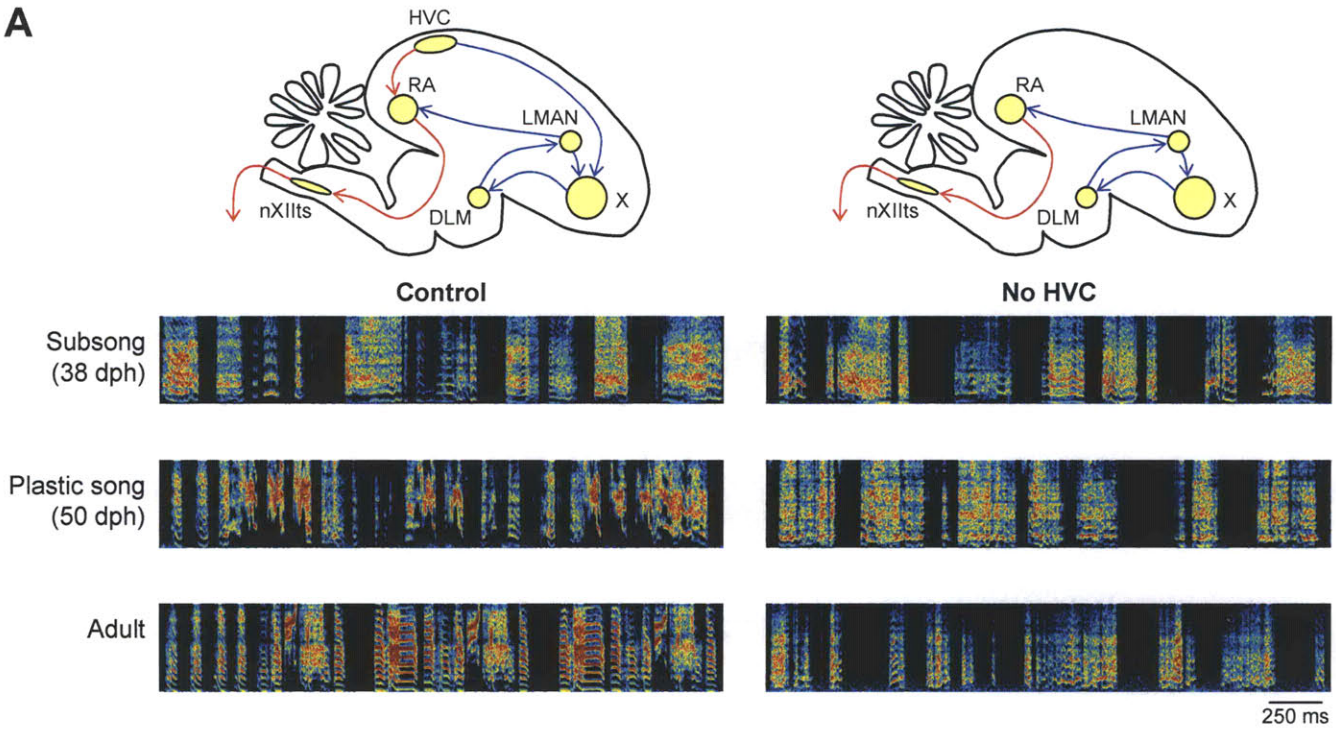
A
HVC
RA
LMAN
X
nXIIts
DLM
Control
No HVC
Subsong
(38 dph)
Plastic song
(50 dph)
Adult
250 ms

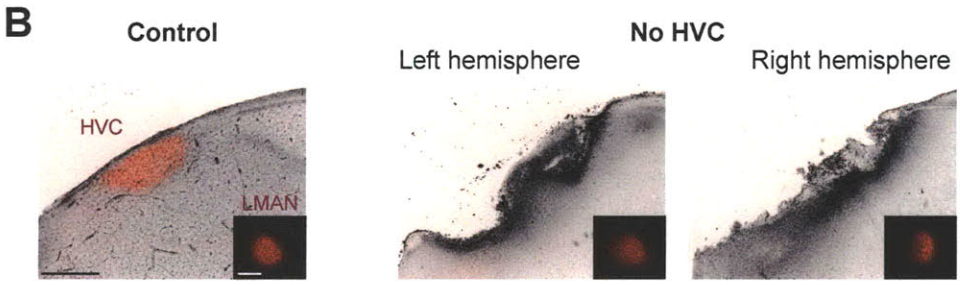
B
Control
No HVC
Left hemisphere
Right hemisphere
HVC
LMAN

Figure 1. Subsong production does not require HVC. (**A**) Results of bilateral HVC elimination (by lesion or pharmacological inactivation). Top: major connections of the song system with and without HVC. Red, motor pathway; blue, anterior forebrain pathway (AFP); X, area X, a basal-ganglia homolog; DLM, dorsolateral nucleus of the anterior thalamus; nXIIts, tracheosyringeal portion of the hypoglossal nucleus. Lower left: Sonograms of three birds at different ages. Lower right: Sonograms of the same birds in the absence of HVC. Frequency ranges from 500 Hz to 7.5 kHz; color scale (from black to red) spans a power range of 8 dB. For audio clips of these songs, see supplementary information. (**B**) Histological verification of HVC lesions. Left: Inverted dark-field image of a parasagittal section of a normal zebra finch brain (50 dph). Red indicates retrograde fluorescence labeling of neurons in HVC after tracer (Alexa-conjugated cholera toxin subunit β) injection into RA. Inset: retrograde labeling of neurons in LMAN from the same injection. Right: Brain sections of the plastic-song bird shown in (A). Scale bars, 500 μm.

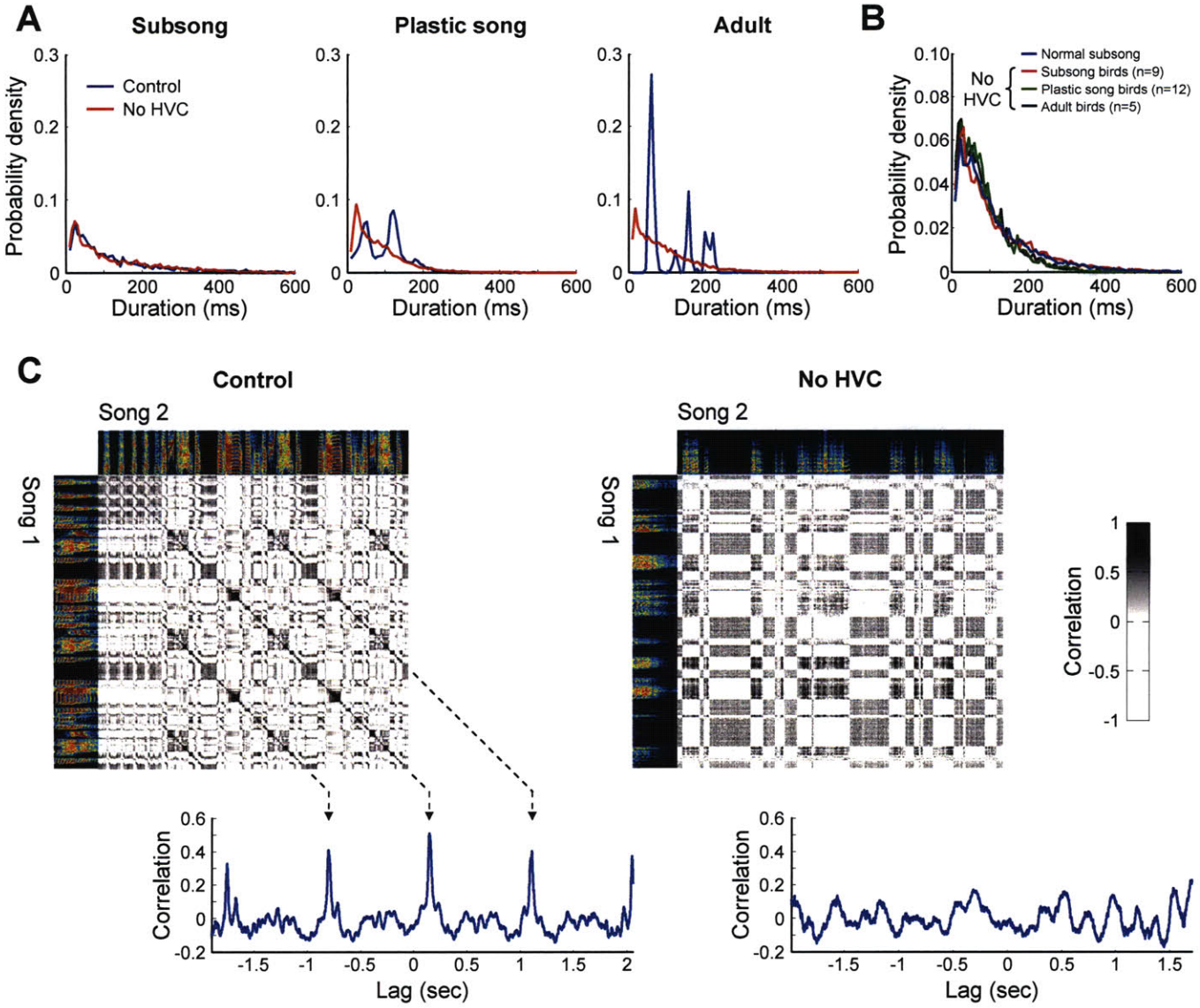
A
Subsong
Plastic song
Adult
Control
No HVC
Probability density
Duration (ms)
B
Normal subsong
Subsong birds (n=9)
Plastic song birds (n=12)
Adult birds (n=5)
No HVC
C
Control
No HVC
Song 2
Song 1
Correlation
Lag (sec)
D
control
no HVC
Correlation
Age (dph)
Adult

Figure 2. Singing in the absence of HVC is highly similar to normal subsong. (**A**) Distributions of syllable durations for three birds of various ages (blue) and distributions for the same birds in the absence of HVC (red). (**B**) Average syllable duration distributions for normal subsong-producing birds (blue) and birds of different ages in the absence of HVC. (**C**) Sample spectral correlation matrices for a pair of songs produced by an adult bird (left) and by the same bird after HVC lesion (right). Averaging the matrix along its diagonals reveals strong correlation peaks in control (pre-lesion) condition, but not after HVC lesion. (**D**) Maximum values of the spectral correlation, averaged across all pairwise comparisons of 10 song bouts (see supplementary information), for birds in control conditions and for the same birds in the absence of HVC. Dashed lines, linear regression; error bars, SEs across all 45 pairwise comparisons.

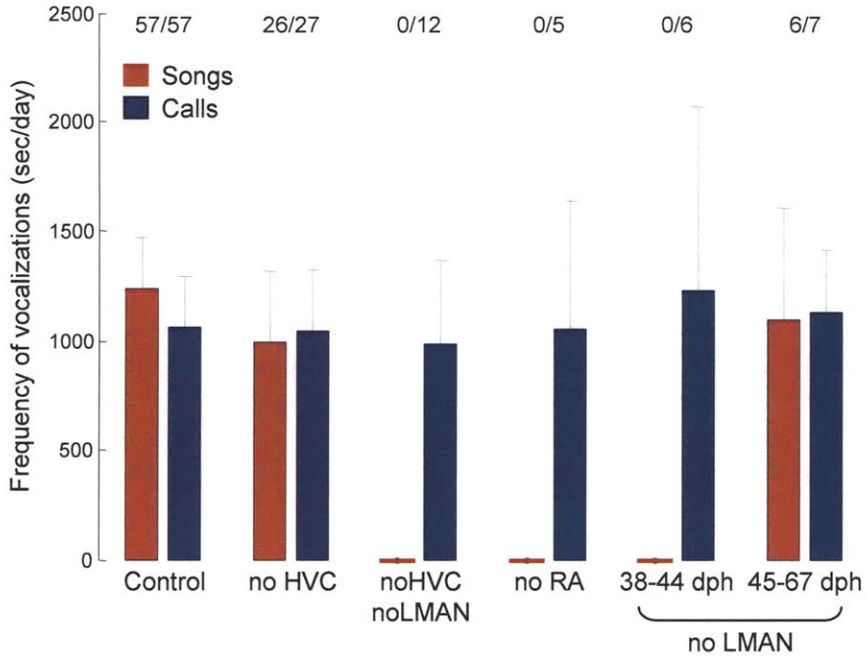
57/57
26/27
0/12
0/5
0/6
6/7
Songs
Calls
Frequency of vocalizations (sec/day)
2500
2000
1500
1000
500
0
Control
no HVC
noHVC
noLMAN
no RA
38-44 dph
45-67 dph
no LMAN

Figure 3. Subsong production requires LMAN and RA. Average rates of song and call production in all lesion and inactivation experiments are shown. For rate measurement, a full day of recording was partitioned into 1-s segments, and the numbers of segments containing calls or songs were estimated (see supplementary information). In cases where age is unspecified, data from all birds are pooled together. Note that for subsong-producing birds (<45 dph), the average rate of singing was not affected by HVC elimination (Wilcoxon $P > 0.5$). LMAN lesions in older juveniles (rightmost group) resulted in highly stereotyped song (Ölveczky, Andalman et al. 2005). Values at top are fractions of experiments in which any amount of singing occurred. Error bars are SEM values across birds. In experiments that abolished singing, silencing was specific to songs and did not affect the frequency of call vocalizations that are known not to require the song system (Simpson and Vicario 1990).

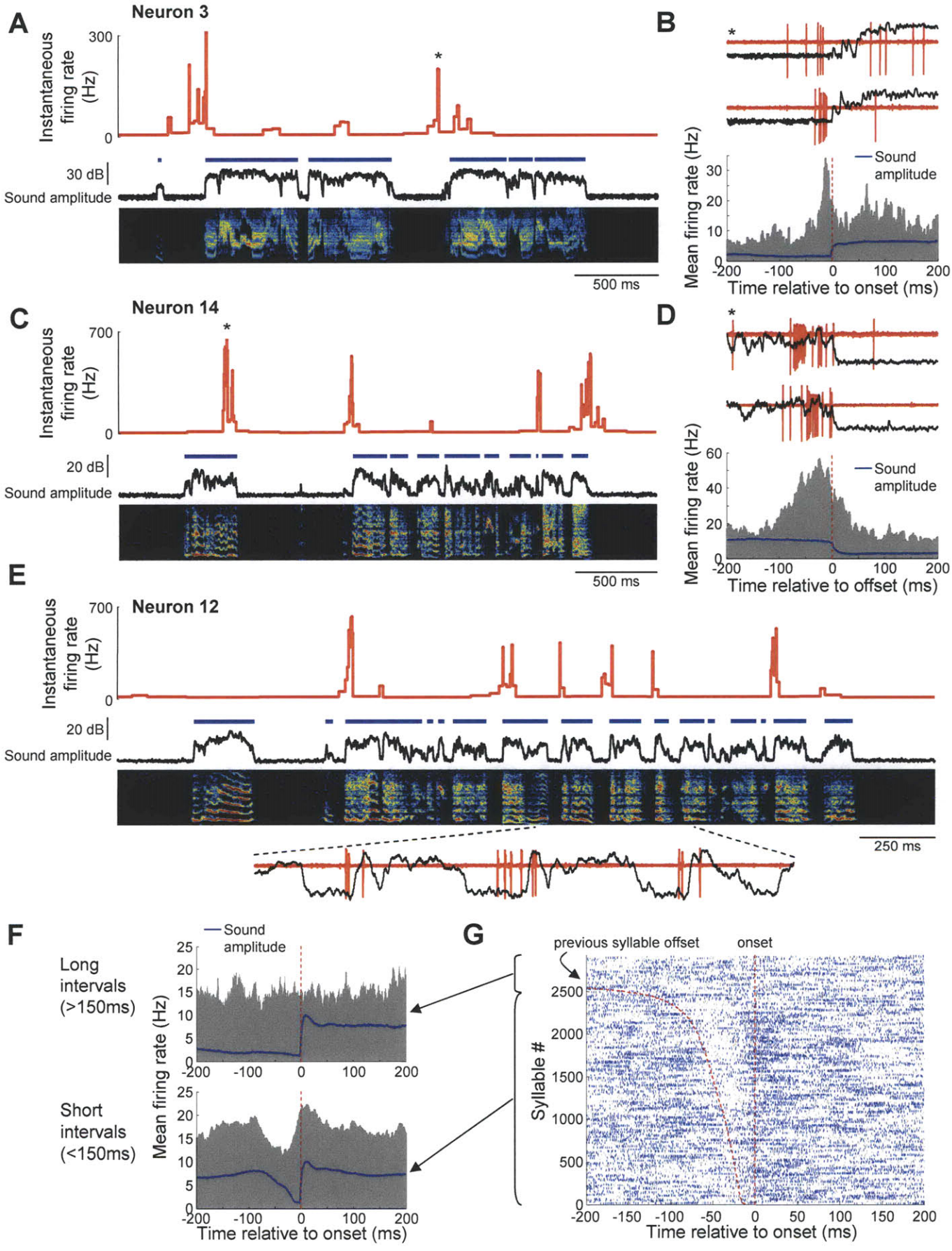
A
Neuron 3
Instantaneous firing rate (Hz)
300
0
30 dB
Sound amplitude
500 ms
B
Mean firing rate (Hz)
Sound amplitude
Time relative to onset (ms)
C
Neuron 14
700
20 dB
D
Time relative to offset (ms)
E
Neuron 12
250 ms
F
Long intervals (>150ms)
Short intervals (<150ms)
G
previous syllable offset
onset
Syllable #
2500
2000
1500
1000
500

Figure 4. LMAN exhibits premotor activity during subsong. (**A**) Activity of an RA-projecting LMAN neuron during subsong production. Blue segments indicate individual syllables. Instantaneous firing rate exhibits peaks before syllable onsets. (**B**) Examples of spiking activity (red) before onset of sound amplitude (black) for neuron 3. Asterisk indicates a matching example with (A). Histograms show average firing rate across all syllable onsets for neuron 3; blue trace, average sound amplitude. Average includes only those syllables that were preceded by long (>150 ms) periods of silence. (**C** and **D**) Activity of a neuron that exhibited peaks in firing before syllable offsets, plotted as in (A) and (B). Averages in (D) include only long (>150 ms) syllables that were followed by long (>150 ms) periods of silence in order to isolate offset-related changes in firing from onset-related changes. (**E**) Activity of a neuron that exhibited firing before syllable onsets after short (<150 ms) intervals, plotted as in (A). Bottom: Spiking activity (red) occurring before syllable onsets for neuron 12. (**F**) Averages of firing rate and sound amplitude for neuron 12, separately for syllables that followed short (10 to 150 ms) and long (>150 ms) intervals, plotted as in (B). (**G**) Syllable onset–centered spike raster for neuron 12. Raster is sorted according to the length of the interval that preceded the syllables; dashed lines indicate interval boundaries. Blue marks, spikes that occurred in high-frequency (>100 Hz) bursts; gray marks, spikes that occurred outside of bursts.

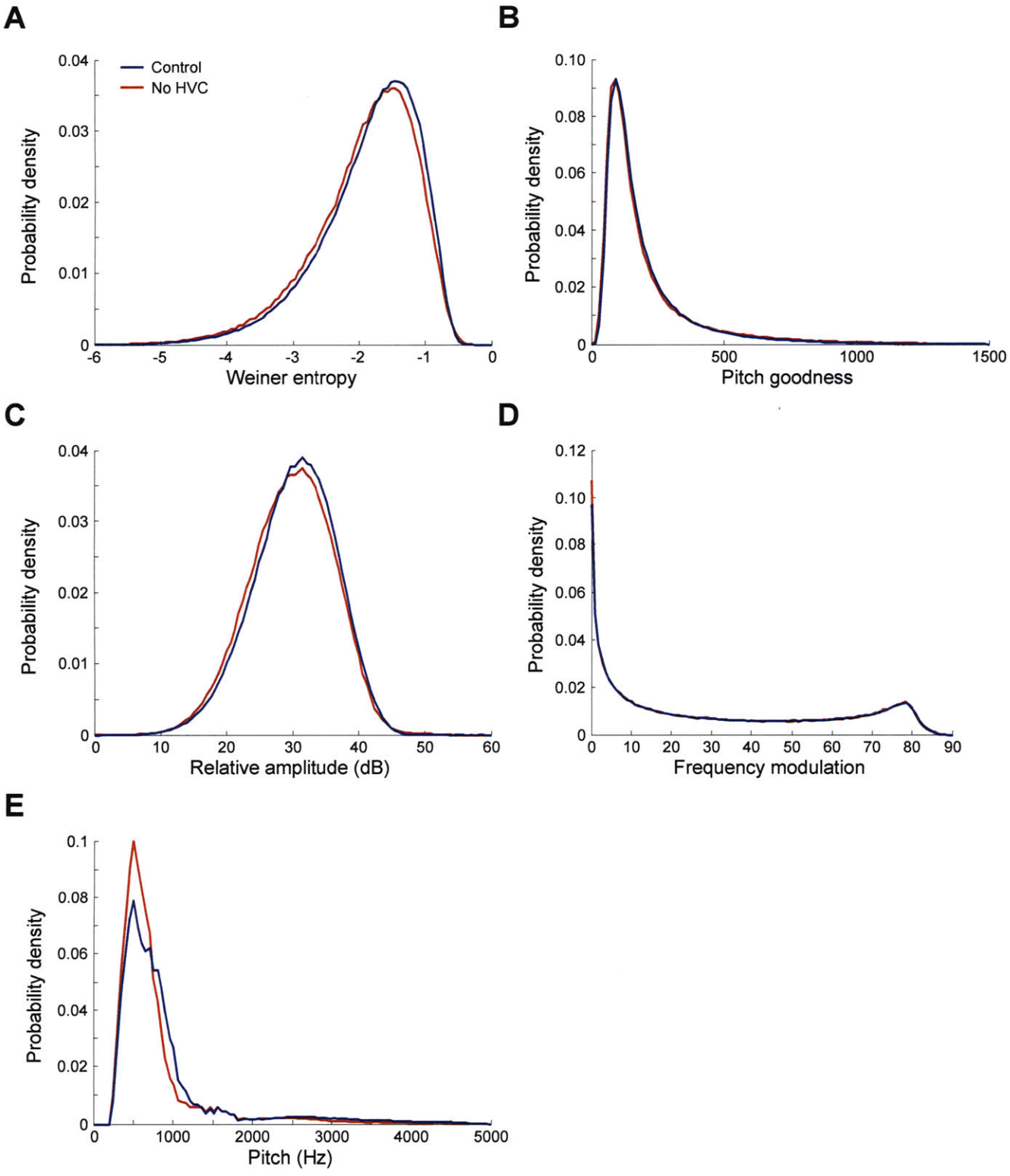
A
Control
No HVC
Probability density
0.04
0.03
0.02
0.01
0
-6
-5
-4
-3
-2
-1
0
Weiner entropy
B
Probability density
0.10
0.08
0.06
0.04
0.02
0
0
500
1000
1500
Pitch goodness
C
Probability density
0.04
0.03
0.02
0.01
0
0
10
20
30
40
50
60
Relative amplitude (dB)
D
Probability density
0.12
0.10
0.08
0.06
0.04
0.02
0
0
10
20
30
40
50
60
70
80
90
Frequency modulation
E
Probability density
0.1
0.08
0.06
0.04
0.02
0
0
1000
2000
3000
4000
5000
Pitch (Hz)

Figure S1. Singing in the absence of HVC is highly similar to normal subsong. (a-e) Average distributions of acoustic features (see text) before and after elimination of HVC across 9 birds under the age of 45 dph.

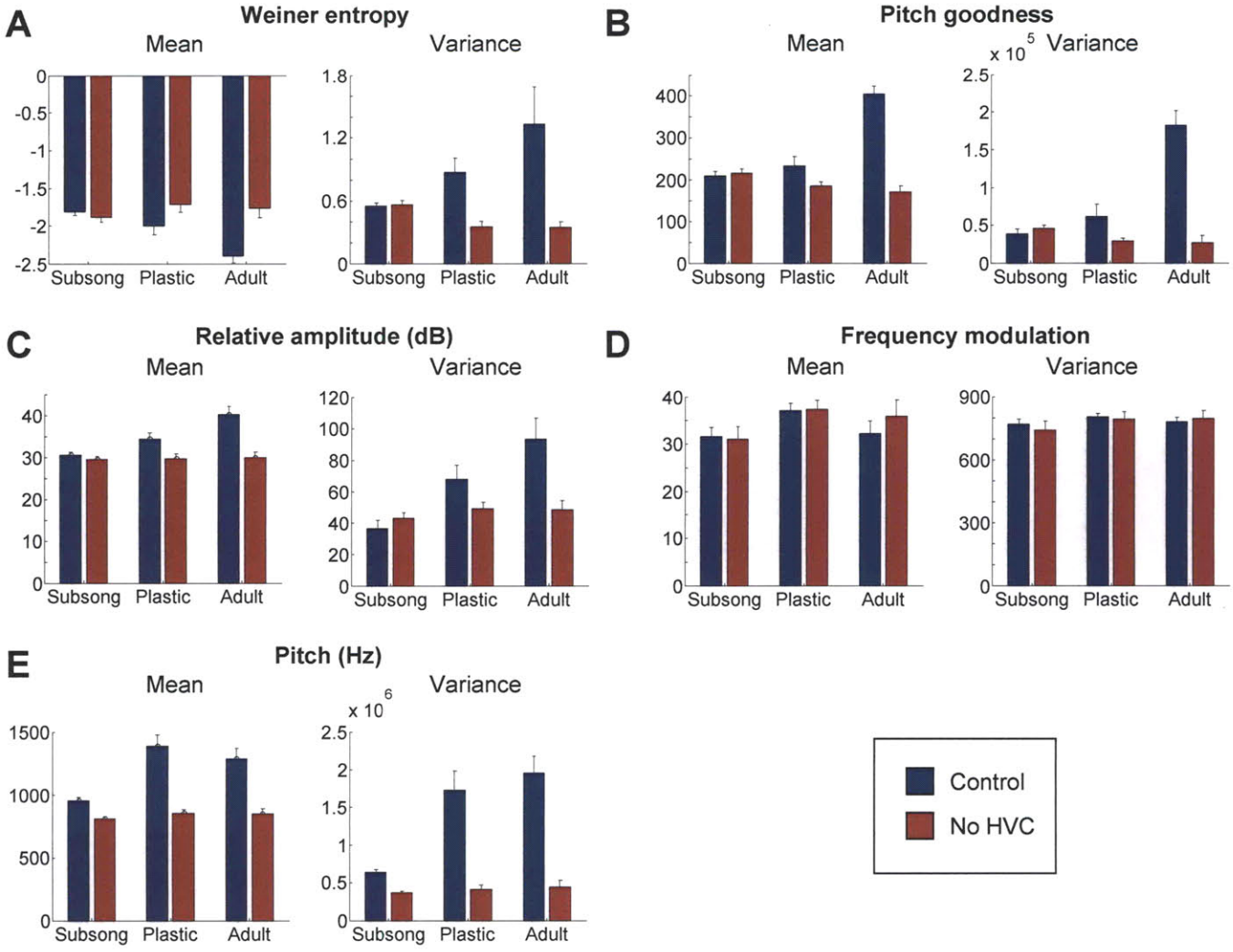
A
Weiner entropy
Mean
Variance
B
Pitch goodness
Mean
x 10^5 Variance
C
Relative amplitude (dB)
Mean
Variance
D
Frequency modulation
Mean
Variance
E
Pitch (Hz)
Mean
Variance
x 10^6
Subsong
Plastic
Adult
Control
No HVC

Figure S2. Quantification of acoustic features (see text) before and after HVC elimination for three age groups: subsong-producing birds (33-44 dph), plastic-song birds (45-73 dph), and adults. (a-e) Means and variances of acoustic features, averaged across all birds in each age group.

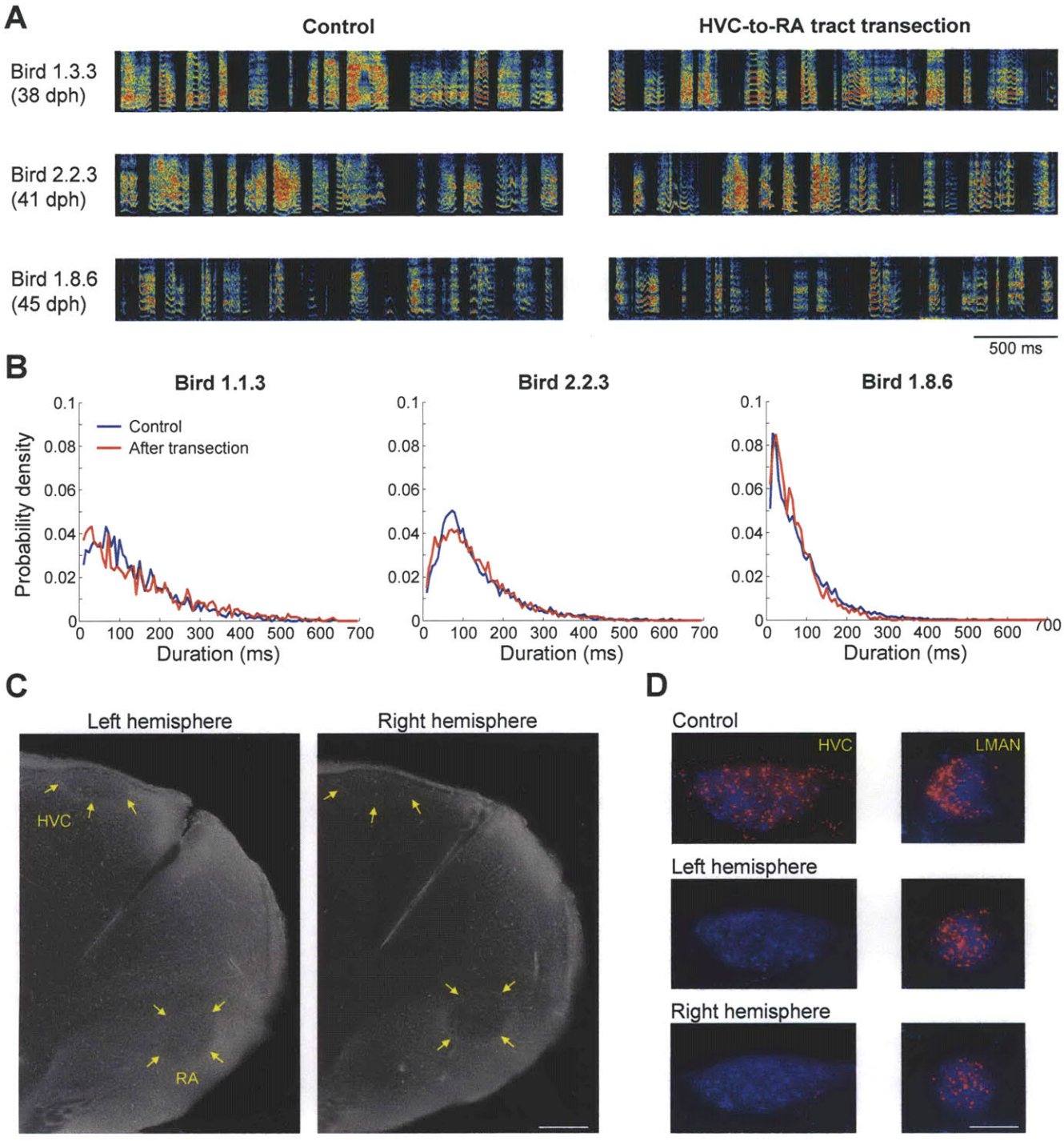
A
Control
HVC-to-RA tract transection
Bird 1.3.3
(38 dph)
Bird 2.2.3
(41 dph)
Bird 1.8.6
(45 dph)
500 ms
B
Bird 1.1.3
Bird 2.2.3
Bird 1.8.6
Control
After transection
Probability density
Duration (ms)
C
Left hemisphere
Right hemisphere
HVC
RA
D
Control
HVC
LMAN
Left hemisphere
Right hemisphere

Figure S3. Singing following transection of the HVC-to-RA fiber tract is highly similar to normal subsong. (a) Sample sonograms of 3 birds in the subsong stage (*left*) and sample sonograms of the same birds following bilateral transection of the HVC-to-RA fiber tract (*right*). (b) Distributions of syllable durations for the 3 birds shown in (a) before and after transection. Distributions in the two conditions are almost entirely overlapping. (c) Parasagittal brain sections of Bird 1.8.6 shown in (a) and (b), illustrating the location and extent of the transections in the two hemispheres. (d) Fluorescence images for the same bird and an age-matched control bird. Retrograde labeling of neurons following tracer injection (alexa-conjugated dextran) into RA (red) is overlaid with retrograde labeling following injection into area X (blue). Both X- and RA-projecting neurons in HVC are labeled in the control bird, but only X-projectors are labeled in the bird that received transections. In both birds, both X- and RA-projecting neurons in LMAN are labeled.

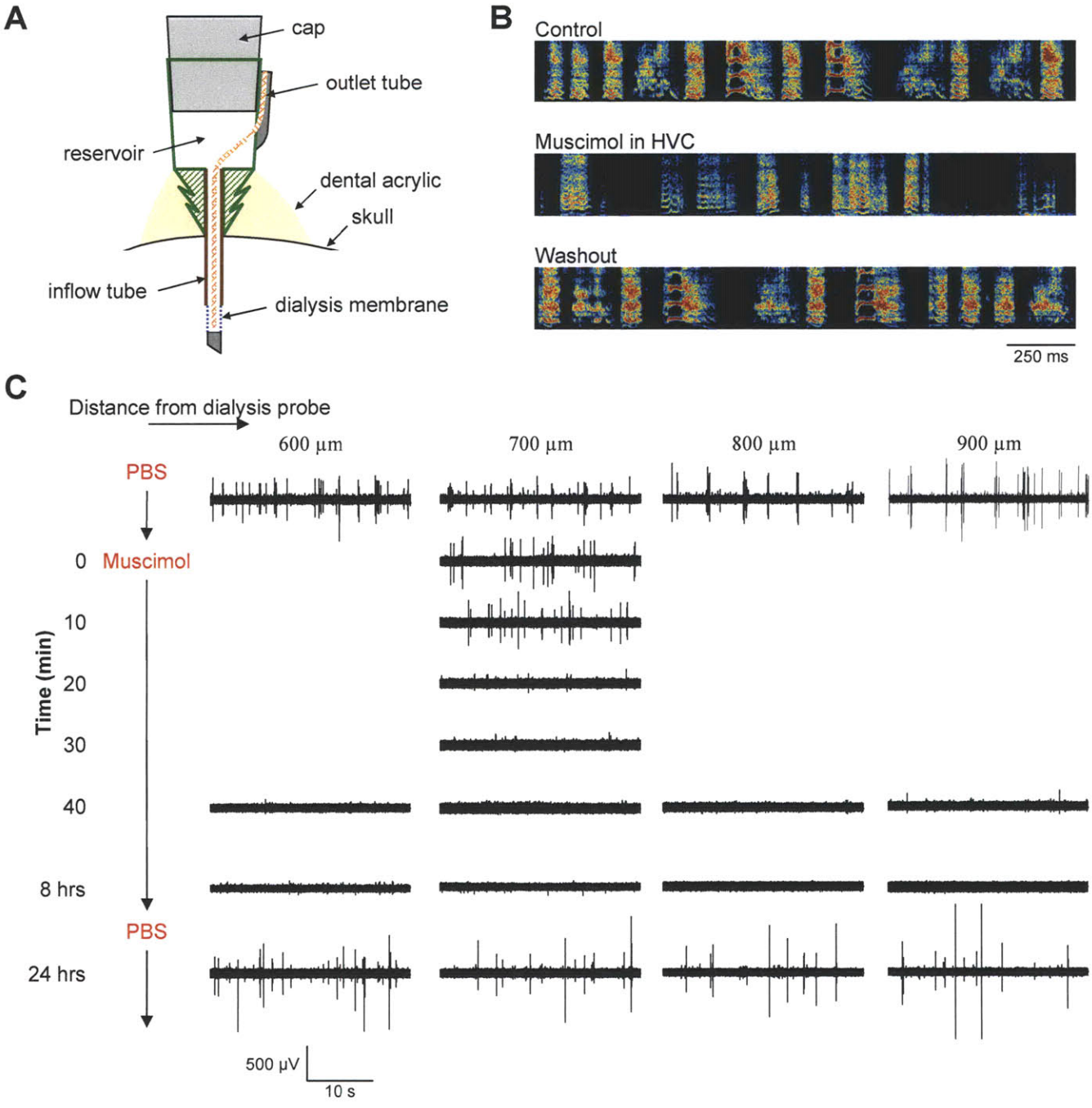
A
cap
outlet tube
reservoir
dental acrylic
skull
inflow tube
dialysis membrane
B
Control
Muscimol in HVC
Washout
250 ms
C
Distance from dialysis probe
600 µm
700 µm
800 µm
900 µm
PBS
0
Muscimol
10
20
30
40
8 hrs
PBS
24 hrs
Time (min)
500 µV
10 s

Figure S4. Bilateral inactivation of HVC in the singing bird. (a) Schematic diagram of an un-tethered reverse microdialysis probe for drug delivery to brain areas in a freely behaving zebra finch. Drug (muscimol or TTX) is placed in the reservoir, from which it freely diffuses along the inflow tube and across the semipermeable dialysis membrane. Outlet tube is used for drug washout. (b) Song production in a 50-day-old bird following bilateral inactivation of HVC with muscimol (1.5 mg/ml) and restoration of normal singing following washout. (c) Confirmation of HVC inactivation by the dialysis probe. Spontaneous activity under anesthesia was recorded at various distances from the probe following application of phosphate-buffered saline (PBS). Muscimol (1.5 mg/ml) was then placed in the probe (0 min) and activity was recorded at a fixed location 700 μm from the probe until 40 min. Spontaneous activity was abolished ~20 min after drug application. After 40 min, the lack of spontaneous activity was confirmed at locations up to 900 μm from the probe. Blockade of activity at these locations was confirmed again in a different recording session 8 hours after drug application. Drug was then washed out by replacement with PBS, and a normal level of spontaneous activity was recorded the following day (24 hrs).

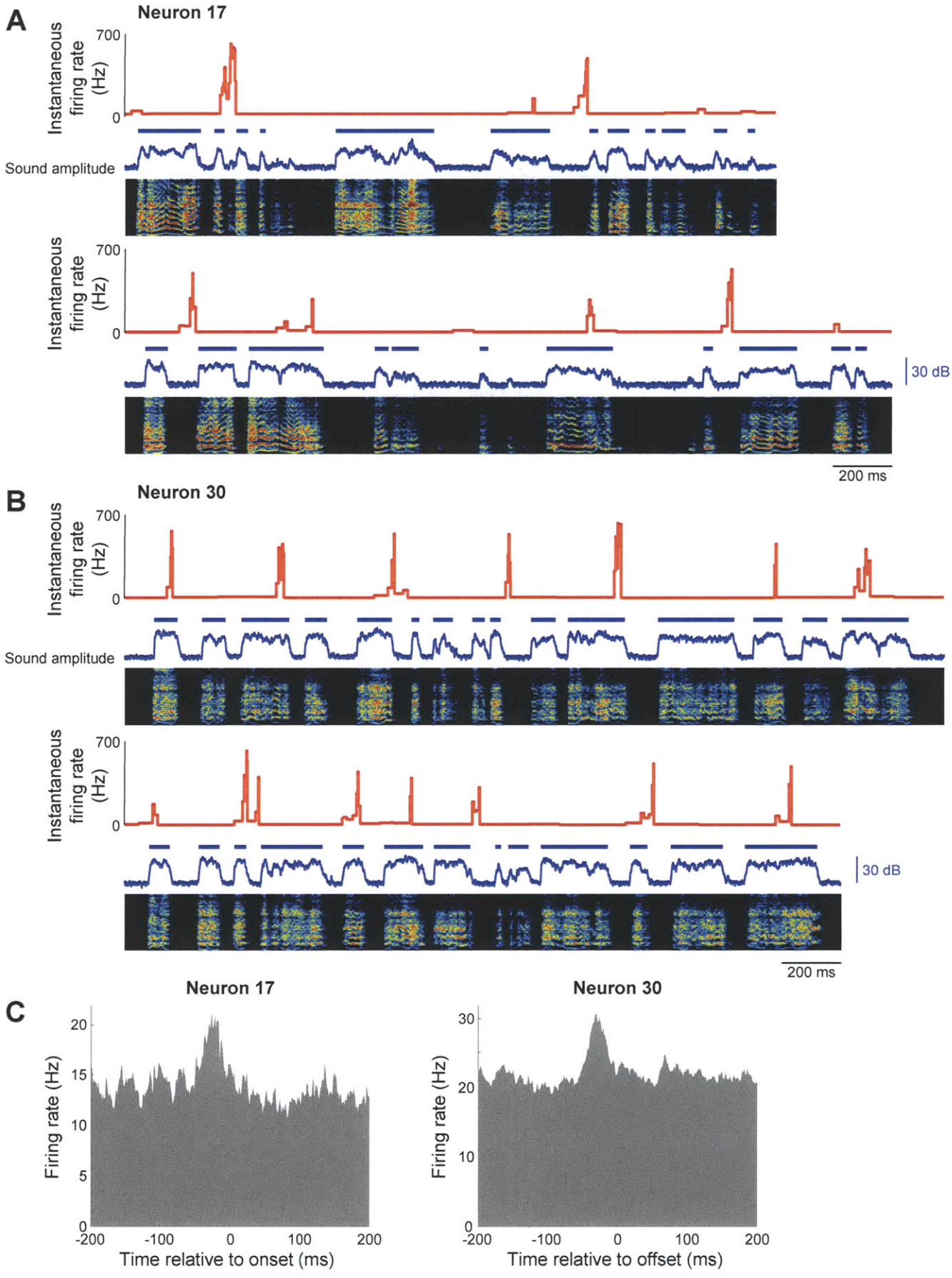
A
Neuron 17
Instantaneous firing rate (Hz)
700
0
Sound amplitude
30 dB
200 ms
B
Neuron 30
C
Neuron 17
Neuron 30
Firing rate (Hz)
Time relative to onset (ms)
Time relative to offset (ms)
-200
-100
0
100
200

Figure S5. LMAN exhibits premotor activity during singing in birds with bilateral HVC lesions. (a) Activity of an RA-projecting LMAN neuron during two consecutive bouts of singing. Blue segments indicate individual syllables. The neuron exhibits increased firing prior to syllable onsets. (b) Activity of an RA-projecting LMAN neuron that exhibits increased firing prior to syllable offsets. Activity during two consecutive bouts of singing is plotted as in (a). (c) Average firing rates across all syllable onsets for the neuron shown in (a) and across all syllable offsets for the neuron shown in (b).

Chapter 4

A basal ganglia-forebrain circuit in the songbird biases motor output to avoid vocal errors

Attributions.

This chapter was previously published as:

Andalman AS and Fee MS, 2009. A basal ganglia-forebrain circuit in the songbird biases motor output to avoid vocal errors. *Proc Natl Acad Sci U S A* 106(30):12518-23.

In songbirds, as in mammals, basal ganglia-forebrain circuits are necessary for the learning and production of complex motor behaviors, however the precise role of these circuits remains unknown. It has recently been shown that a basal ganglia-forebrain circuit in the songbird, which projects directly to vocal-motor circuitry, has a premotor function driving exploration necessary for vocal learning. It has also been hypothesized that this circuit, known as the anterior forebrain pathway (AFP), may generate an instructive signal that improves performance in the motor pathway. Here we show that the output of the AFP directly implements a motor correction that reduces vocal errors. We use disruptive auditory feedback, contingent on song pitch, to induce learned changes in song structure over the course of hours and find that reversible inactivation of the output of the AFP produces an immediate regression of these learned changes. Thus, the AFP is involved in generating an error-reducing bias, which could increase the efficiency of vocal exploration as well as instruct synaptic changes in the motor pathway. We also find that learned changes in the song generated by the AFP are incorporated into the motor pathway within one day. Our observations support a view that basal ganglia-related circuits directly implement behavioral adaptations that minimize errors and subsequently stabilize these adaptations by training premotor cortical areas.

Birdsong is a complex motor behavior which, like many human motor skills, improves with practice. Songbirds learn to sing by imitation, using auditory feedback to compare their own vocalizations with the memorized song of a tutor (Konishi 1965). Learning birds initially produce a highly variable juvenile song that, after thousands of repetitions, converges to a stable adult song, often a remarkably precise imitation of the tutor song (Immelmann 1969; Tchernichovski, Lints et al. 1999). Like many motor learning tasks in mammals (Knowlton, Mangels et al. 1996; Wise, Murray et al. 1996; Graybiel 2008), this goal-directed behavior requires a basal ganglia-thalamocortical

circuit (Fig. 1A) known as the anterior forebrain pathway (AFP) (Bottjer, Miesner et al. 1984; Sohrabji, Nordeen et al. 1990; Scharff and Nottebohm 1991; Brainard and Doupe 2000; Farries and Perkel 2002). The mechanisms by which basal ganglia circuitry support motor learning are largely unknown, but evidence suggests that the basal ganglia are necessary to express recently learned behavior (Atallah, Lopez-Paniagua et al. 2007), and that changes in neural activity in response to learning appear first in basal ganglia circuits (Pasupathy and Miller 2005; Yin, Mulcare et al. 2009).

It has recently been shown that the AFP plays a premotor role in driving vocal variability. Inactivation or lesions of LMAN (lateral magnocellular nucleus of the nidopallium), the output pathway of the AFP, largely eliminate song variability in both juvenile (Scharff and Nottebohm 1991; Ölveczky, Andalman et al. 2005) and adult birds (Kao, Doupe et al. 2005). Furthermore, single-unit recordings in young birds show that LMAN neurons projecting to the motor pathway exhibit highly variable bursts of activity immediately preceding modulations in vocal output (Aronov, Andalman et al. 2008). Finally, electrical stimulation of LMAN can produce transient changes in song amplitude or pitch (Kao, Doupe et al. 2005). Thus, LMAN neurons projecting to the motor pathway exert a direct premotor influence on vocal output, driving vocal variability that can be used for learning (Tumer and Brainard 2007). These results support the hypothesis that vocal learning proceeds by trial-and-error (or reinforcement) learning (Doya and Sejnowski 1998; Troyer and Doupe 2000; Ölveczky, Andalman et al. 2005; Fiete, Fee et al. 2007; Tumer and Brainard 2007).

It has been proposed that the AFP, in addition to generating variability, evaluates vocal errors and transmits a signal to guide plasticity in the motor pathway (Bottjer,

Miesner et al. 1984; Troyer and Bottjer 2001). This hypothesis is supported by the fact that lesions of the AFP prevent changes in juvenile (Scharff and Nottebohm 1991) and adult (Brainard and Doupe 2000) song. Given the premotor influence of the AFP, an interesting possibility is that this hypothesized signal takes the form of a premotor drive that biases the song away from vocal errors. It has been suggested that such a premotor bias could instruct long-term plasticity in the motor pathway (Troyer and Doupe 2000). Here we combine two techniques – experimentally controlled vocal learning and transient localized brain inactivation – to demonstrate that the AFP is involved in generating a corrective premotor bias.

Results

Natural song learning proceeds slowly and unpredictably, and it is difficult to quantitatively associate song changes with a reduction in perceived vocal error. To make vocal learning more experimentally accessible, we developed a training protocol in which we controlled vocal learning by manipulating auditory feedback, similar to a recently described approach (Tumer and Brainard 2007). Disruptive auditory feedback was played to the bird during singing (Leonardo and Konishi 1999) using an implanted speaker, and was made contingent on the pitch of a region within a targeted song syllable (Fig. 1A and B, see supporting information (SI)). Playing disruptive auditory feedback during moments when the pitch of the targeted syllable was above a threshold value caused the bird to sing the targeted syllable at gradually lower pitches (Fig. 1C and D). Upward movement of pitch was produced by reversing the contingency (Fig. 1D). In order to induce many sequential days of learning, we re-centered the threshold of the disruptive

auditory feedback each morning before the bird awoke. The average pitch of the targeted syllable changed most rapidly during the first four hours after waking, at a rate of 3.5±0.4 Hz/hour (Fig. 1E, n = 5 birds, 80 experimental days, s.e.m. unless otherwise indicated) in the instructed direction. The induced changes in pitch resulted in a reduction in the amount of disruptive auditory feedback (Fig. 1F), quantified as the average power of the feedback played during the targeted syllable. While the majority of pitch changes in the targeted syllable occurred during the day, the pitch in the morning had a tendency to begin beyond what was acquired by the end of the previous day (Fig. 1G, 5.86±1.4 Hz in the instructed direction, $p < 0.01$, two-tailed t-test). For comparison, the average total shift during the day was 15.5±1.5 Hz in the instructed direction (Fig. 1H, $p < 10^{-14}$). This change in pitch was larger, on average, than the standard deviation of the pitch of the targeted syllable (by 144±13%); thus, to summarize, our protocol produced learned changes in pitch over the course of several hours that dramatically reduced the amount of auditory feedback 'error.'

Does the AFP make a premotor contribution to these adaptive changes in pitch? To address this question, we used tetrodotoxin (TTX) to transiently inactivate LMAN during learning (Fig. 2A and Fig. S3 and S4). If premotor signals from the AFP directly contribute to learned pitch changes, we would expect inactivation to cause an immediate regression of the learned change. In contrast, if the learned pitch changes are immediately implemented by synaptic plasticity in the motor pathway, we would expect the average pitch to be the same before and after LMAN inactivation. Each day, after the first 4 hours of conditional feedback, we infused either TTX or vehicle (on alternating days) into LMAN. Inactivation of LMAN with TTX produced an immediate change in pitch in a

direction opposite that of the ongoing learning (Fig. 2B and E, 16.3±2.2 Hz, n = 34 inactivations, $p < 10^{-7}$, two-tailed t-test, also see Fig. S5 and S6). Inactivation most often resulted in an increase in the amount of disruptive auditory feedback played to the bird (Fig. 2D, 30/34 inactivations, feedback power increased by a factor of 15±6, $p<0.02$, one-tailed t-test). In contrast, infusion of vehicle had no significant effect on average pitch (Fig. 2C and F, n = 34 infusions, $p > 0.4$). These observations suggest that the AFP contributes to vocal output by biasing syllable pitch in a direction that reduces experimentally imposed 'error'.

In the remaining text, we refer to the pitch of the targeted syllable generated with LMAN intact and LMAN inactivated as 'LMAN(+) pitch' and 'LMAN(-) pitch' respectively. The motor pathway encodes a stereotyped motor program for singing that operates independently of the AFP (Bottjer, Miesner et al. 1984; Scharff and Nottebohm 1991; Kao, Doupe et al. 2005; Ölveczky, Andalman et al. 2005). Thus, LMAN(-) pitch reflects this motor-pathway-encoded version of the targeted syllable. Furthermore, the contribution of the AFP to the pitch of the targeted syllable can be quantified as LMAN(+) pitch minus LMAN(-) pitch, which we refer to as 'AFP-dependent bias,' or more briefly as 'AFP bias.'

Across multiple days of conditional feedback, we observed large accumulating changes in the pitch of the targeted syllable (Fig. 1D). Are all of these changes due to accumulating AFP bias, or is there also a contribution from AFP-independent mechanisms, such as plasticity in the motor pathway? To answer this question, we examined whether, over the course of the experiment, LMAN(-) pitch deviated from the baseline pitch sung prior to the first day of conditional feedback. We found that LMAN(-

) pitch exhibited substantial deviations from baseline (Fig. 3A and B, range = 121±5 Hz s.d.). In fact, deviations in LMAN(-) pitch closely tracked the deviations of LMAN(+) pitch (Fig. 3C and D, $r^2 = 0.85$, slope = 1.08±0.08), indicating that during the course of our experiments, AFP-independent mechanisms contributed substantially to the learning. In contrast, the magnitude of the AFP bias was not correlated with how far the pitch deviated from baseline (Fig. 3E, $p > 0.3$, slope not different from 0), indicating that continued learning in the same direction over multiple days was not associated with increasing AFP-dependent bias, but rather with an increasing AFP-independent contribution.

Consistent with this result, we found that LMAN inactivation caused the pitch of targeted syllable to regress to near the pitch in the first morning songs. That is, the size of the AFP bias was correlated with the size of the pitch shift that occurred during the morning prior to inactivation (Fig. 4A and B, $r^2=0.73$, slope=0.98±0.11, $p<10^{-9}$, slope greater than zero). Taken together, our findings thus far suggest that the AFP makes a direct contribution to learned pitch changes, but that the size of the AFP contribution is limited and is proportional to the amount of learning that occurred within the last day. In addition, during multiple days of learning, the majority of the accumulated deviations in pitch from baseline are encoded in the motor pathway.

The syllable pitch encoded in the motor pathway is highly plastic, as evidenced by the large changes in LMAN(-) pitch between successive inactivations (Fig. 3B). We tested the hypothesis that these plastic changes are predicted by the amount of AFP bias observed during some preceding interval. Plastic changes in the motor pathway were assessed as the difference between successive LMAN(-) syllable pitch measurements

(Fig. 5A). Because LMAN inactivations were carried out every other day, this measure reflects the change over a two-day interval. The total AFP bias was also calculated in two-day intervals as a sum over consecutive days. AFP bias was directly measured on TTX days, and was estimated on vehicle days as the total amount of learning that occurred on that day — motivated by our finding that the AFP bias is roughly equal to the amount of learning that occurred prior to TTX infusion (Fig. 4).

We started by examining the relation between motor pathway plasticity and AFP bias one day earlier. Specifically, we find that plastic changes in the motor pathway over a two day interval (e.g. between day n-2 and n) are strongly correlated with the sum of the AFP bias measured on day n-2 and estimated on day n-1 (Fig. 5A-C, $r^2 = 0.93$, slope = 0.99±0.06). This correlation is further supported by examining *variations* in the amount of learning. On up days, a large AFP bias was typically followed by a large change in the motor pathway, and a small AFP bias was followed by a small change. This same relation held for down days, but the signs of the quantities were negative. To combine all the data, we inverted the sign of the data for down days. We find that variations in the AFP bias were correlated with variations in motor plasticity observed one day later (Fig. 5D, lag = -1 day: r^2=0.70). These findings suggest a strong link between AFP bias and plasticity in the motor pathway within the next day.

We next examine the temporal specificity of the relation between in AFP bias and subsequent plasticity in the motor pathway. Do variations in AFP bias only correlate with changes in LMAN(-) pitch on the next day, or do they also correlate with motor plasticity on the same day, or two days later? We find that variations in AFP bias were not as strongly correlated with variations in motor plasticity observed on the same day

(lag = 0, r^2=0.18), or two days later (Fig. 5D and E, lag = -2: r^2=0.08, also see Fig. S7). Of course, a careful examination of the temporal relation between AFP bias and plasticity in the motor pathway will require experiments in which LMAN inactivations are carried out at a finer time resolution. Nevertheless, these results lend further support to the hypothesis that AFP bias may be subsequently consolidated in the motor pathway by the end of the following day.

AFP bias was not only correlated with plastic changes in the motor pathway over the next day, but these quantities have the same magnitude. The change in LMAN(-) pitch between successive measurements (average 16.7±1.2 Hz/day) was not significantly different from the size of the estimated AFP bias at a lag of minus one day (average 15.1±1.7 Hz, $p > 0.11$, paired t-test, see Methods), suggesting a remarkable correspondence between the mechanisms that underlie AFP bias and subsequent motor plasticity.

Discussion

Our experiments take advantage of the fact that, in response to an experimentally controlled association between syllable pitch and auditory error, a singing bird makes rapid corrective pitch changes (Tumer and Brainard 2007). Applied over many days this protocol produced large accumulating changes in pitch. By inactivating the AFP during learning, we identified two contributions to the observed vocal plasticity. First, we found an AFP-dependent contribution that biases vocal output to reduce the probability and intensity of imposed error. Second, we observed an AFP-independent contribution, likely due to plasticity in the motor pathway, that accounts for the majority of the accumulated

pitch changes. We also found a remarkable correspondence between AFP-dependent bias and plastic changes in the motor pathway observed within the next day. Thus, our results establish the time scale on which adaptive changes in vocal output become encoded in the motor pathway and expressed in an AFP-independent manner.

Our findings suggest a possible role for AFP-dependent bias during natural vocal imitation. It will be important to determine whether during natural song learning, the AFP biases motor output to improve the match between auditory feedback and tutor song. This could be examined by carrying out LMAN inactivations during the rapid, early phases of vocal learning in juvenile songbirds. We anticipate that inactivation of LMAN in the evening would result in a regression of learned changes in the song that occurred during the hours prior to inactivation and furthermore would result in a decrease in similarity with tutor song.

There are a number of mechanisms by which the bias we observe could be dependent on AFP activity. The AFP could serve a permissive role. For example, it is possible that HVC drives bias, but that its expression is dependent on AFP input to RA. Alternatively, the AFP could serve a direct premotor role in generating bias. Given the premotor contribution of the AFP to generating vocal variability, we favor the interpretation that AFP-driven variability itself becomes biased, generating larger or more frequent variations in the direction of reduced vocal error.

The mechanistic role of the three AFP nuclei in generating bias remains to be determined. Striatal neurons have been shown to be involved in the rapid evaluation of rewarded associations (Pasupathy and Miller 2005) and in the coding of action-specific reward values (Kawagoe, Takikawa et al. 1998; Samejima, Ueda et al. 2005). One

possibility is that 'random' activity patterns in LMAN produce variability in vocal output and are evaluated by basal ganglia homologue Area X. The results of this evaluation could then be sent to LMAN (through pallidal-recipient thalamic nucleus DLM) to reinforce LMAN activity patterns that produced desirable vocal output, thereby resulting in bias. Area X receives an efference copy of activity in LMAN (Vates and Nottebohm 1995; Vates, Vicario et al. 1997) and HVC (Nottebohm, Stokes et al. 1976; Kozhevnikov and Fee 2007), thus placing Area X in a position to evaluate exploratory activity in LMAN in the context of the ongoing song. It is not known, however, whether Area X receives evaluative feedback about song performance either from either auditory areas (Keller and Hahnloser 2009) or from midbrain dopaminergic areas (Gale and Perkel 2006).

Trial and error, or reinforcement, learning requires both exploratory behavior and the evaluation of performance (Sutton and Barto 1998). Biased variability could subserve both of these functions. First, biased variability could make motor exploration more efficient by increasing the speed at which effective motor control parameters are discovered. Second, AFP bias could instruct plasticity in the motor pathway (Troyer and Doupe 2000). While we cannot rule out the possibility that AFP bias and motor plasticity are implemented by separate evaluative mechanisms, our finding that the amount of AFP bias on any one day is correlated with, and has the same magnitude, as the amount of plasticity in the motor pathway within the next day, favors a causal role for the AFP in driving this plasticity. Furthermore, it suggests a view in which plasticity in the motor pathway temporally integrates, or accumulates, a more rapidly learned motor signal expressed by the AFP.

The precise time course and mechanism of consolidation into the motor pathway remains an open question. For example, it is possible that AFP bias actively drives plasticity in the motor pathway during singing by an on-line mechanism, such as Hebbian learning (Houk and Wise 1995). On the other hand, a number of studies have shown that sleep may play an important role in the consolidation of learned skills (Wilson and McNaughton 1994; Dave and Margoliash 2000; Walker, Brakefield et al. 2003; Deregnaucourt, Mitra et al. 2005). In particular, observations of sleep-replay activity in the motor pathway (Dave and Margoliash 2000) have led to the proposal that plasticity in this circuit occurs off-line (Dave and Margoliash 2000), perhaps during sleep. Repeated brief inactivation of LMAN at several time points during the day would help distinguish these hypotheses.

Broadly speaking, our observations shed light on the function of basal ganglia-forebrain circuits in vertebrates, particularly the mechanisms by which practice of complex motor skills results in improved performance, and even more generally, the mechanisms by which goal-directed behaviors become entrained as highly stereotyped sequences or habits (Graybiel 2008).

Material and Methods

Subjects

Juvenile and young adult (age 77-182dph) male zebra finches (*Taeniopygia guttata*) were used. Animals were selected for sufficient singing rates (> 200 song bouts per day), and for songs containing a harmonic stack with a pitch unique within the song

and between 500 and 2000 Hz. All procedures were approved by the Massachusetts Institute of Technology Committee on Animal Care. For surgical procedures see supplementary information.

Song recording

Birds were housed individually in custom sound isolation chambers. Singing was measured using a miniature microphone (WBHC-23910, Knowles Electronics, Inc, 0.08g, Fig. S1A) attached to the bird's head during surgery, providing measurement of song largely independent of position in the cage. Monitoring and recording of song was performed using custom MATLAB (Mathworks, Inc.) software.

Conditional auditory feedback

The auditory signal perceived by the bird during singing were disrupted by playing broadband noise. This disruptive auditory feedback was generated using a hearing aid speaker (EM-23046-CX, Knowles Electronics, 0.21g) and transmitted into the cranial airsac surrounding the cerebellum via an implanted speaker tube (Fig. S1). Sound levels were calibrated individually for each bird (see SI). In this configuration, feedback did not distort the signal recorded by the head-mounted microphone, allowing uninterrupted monitoring of singing.

In our conditional feedback protocol, feedback was contingent on a measure of syllable pitch (Tumer and Brainard 2007). This measure was computed using a set of finite-impulse response (FIR) filters implemented in custom software running on a digital

signal processor (RX8, Tucker-Davis Technologies, Inc.) (Fig. S2 and see SI). The measure of syllable pitch was used to calculate the loudness of the feedback noise relative to the loudness of the ongoing song vocalization. In this way, the bird could not escape feedback simply by singing louder (see SI). The pitch threshold for feedback was constant during each day, and was set each morning to the average pitch of the targeted syllable measured at the end of the previous day (see SI). All feedback powers reported are calibrated acoustic power played into the cranial airsac.

Transient LMAN inactivation

Custom reverse microdialysis probes were built using dialysis tubing (200um diameter) attached to a drug reservoir (Fig. S3A and B), as described previously (Aronov, Andalman et al. 2008). Probes were implanted bilaterally into LMAN using stereotaxic coordinates (Fig. S3C). Inactivation was carried out by filling the reservoir and dialysis tubing with 25 μM TTX. This concentration was found to saturate the reduction of pitch variability resulting from inactivation of LMAN (from dose response curve, Fig. S4C). After nominally four hours, drug was removed by flushing the tubing and filling the reservoir with phosphate buffered saline (PBS). On vehicle days, the same procedures were carried out with PBS instead of TTX. Electrophysiological recordings in anesthetized birds (n=2) confirmed that 25 μM TTX infusion completely suppressed electrical activity within 0.75 mm of the probe, thus fully encompassing LMAN. In constrast, activity persisted in medial MAN (1.05 mm from the probe) for the duration of the recording session, at least 1-1.5 hours following infusion.

Experimental design

The birds were housed individually in custom sound-attenuated chambers equipped with 10-channel commutators (SL-88, Dragonfly Inc.), and were maintained on a 12h:12h light-dark cycle. Conditional feedback was started once the birds consistently sang following infusion. TTX and vehicle were infused on alternate days, nominally four hours (3.9±1.0 s.d.) after the first morning singing. Before lights on, the FIR filters were updated so that the edge of the band of targeted pitches (i.e. the threshold) was placed approximately at the center of the pitch distribution at the end of the previous day (last 50 syllables, Fig. S2C). On the morning after a TTX day, the threshold was centered to the average of the last 50 syllables prior to TTX infusion. See supplemental information for further details and statistics. Conditional feedback remained on throughout the entire day, including during and after drug infusion.

Data analysis

Song was segmented into syllables based on song amplitude. The fidelity of the head-mounted microphone signal made segmentation highly reliable. Acoustic feature vectors were calculated for all segmented syllables (duration, amplitude, entropy, pitch goodness, as well as variance of the last two measures). Syllables were classified using these feature vectors and custom hand-clustering software. For each rendition of the targeted syllable, the time course of the pitch (Fig. S5) and feedback power were computed. By averaging this time course, the mean pitch and mean feedback power of each rendition was calculated.

For each experimental day, pitch and feedback power were computed at four key time points during the day by averaging the first 50 renditions of the morning, the last 50 renditions prior to infusion, the first 50 renditions after infusion, and the final 50 renditions of the day. These values were used to quantify the changes in pitch: a) during learning prior to infusion, b) as a result of infusion, and c) overnight. Overnight changes in pitch were quantified as the difference between the morning pitch and that of the final renditions of the preceding day; nights following TTX infusion were excluded from this analysis. Baseline pitch was computed as the average pitch of the targeted syllable on the day prior to starting feedback.

The time series of motor pathway plasticity (Δm) and the estimated AFP bias ($\beta+\beta^*$) were plotted (Fig. 5C) as follows: The motor pathway plasticity plotted on day n was calculated as LMAN(-) pitch on day n minus the LMAN(-) pitch on day n-2. These points appear every other day because LMAN was only inactivated on alternate days. The running two-day sum of AFP bias plotted on day n was calculated as the sum of AFP bias on day n-2 and the AFP bias on day n-1.

Histology

Animals were deeply anesthetized with pentobarbital and perfused with 4% paraformaldehyde (Sigma). Brains were removed from the skull and post-fixed in 4% paraformaldehyde. Brains were sectioned parasagitally with a vibrating microtome (100μm thick, Vibratome 1000, TPI Inc.), and the location of the dialysis membrane was determined (Fig. S3C).

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SUPPLEMENTARY INFORMATION

METHODS

Surgery

Animals were anesthetized with 1.5-2% isoflurane in oxygen and placed in a stereotaxic apparatus (MyNeuroLabs, Inc.) with a head angle of 20 degrees (anterior skull relative to horizontal). LMAN was localized stereotaxically relative to the bifurcation of the sagittal sinus (5.2mm anterior, 1.75mm lateral). Untethered microdialysis probes were implanted bilaterally into LMAN (Fig. 2A and S3) such that the center of the microdialysis tube was located at a depth of 1.82mm from the brain surface, and secured in place with dental acrylic (Flow-It ALC, Pentron Clinical Technologies). The speaker tube for distorted auditory feedback (MicroRenathane Implantation Tubing, Braintree Scientific, Inc., 0.025" O.D., 4 mm length) was surgically inserted through a small hole in the skull into the airsac surrounding the cerebellum (Fig. S1), and sealed into place with dental acrylic. This airsac is continuous with the interior surface of both eardrums. The sound levels in the speaker/airsac system were calibrated by temporarily sealing a small probe microphone (ER-7C, Etymotic Research) into the cranial airsac on the opposite hemisphere. A full transfer function was computed between the speaker drive current and airsac sound level as a function of frequency. After surgery the animal was allowed to recover for several days. The total weight of the implant was 1.6g.

Conditional auditory feedback

In our conditional feedback protocol, the playback of disruptive noise was a function of a measure of syllable pitch. Specifically, the power of the feedback played to the bird as a function of time, $P_F(t)$, was determined by a scalar function, $R(t)$, of the spectral properties of the song as follows: $P_F(t) = \alpha[R(t) - \lambda]_+ P_S(t)$, where $P_s(t)$ is the amount of power in the song vocalization at time t, α is a multiplicative factor that sets the loudness of the feedback relative to the song power, and λ is a threshold value (typically set to 0.85) of the scalar $R(t)$. The brackets $[\ ]_+$ indicate that negative values of the argument were set to zero, so that feedback is only played when the scalar function, $R(t)$, is greater than the threshold λ . $P_s(t)$ was calculated as a low-pass filtered version of the squared audio signal. $P_F(t)$ was computed on-line using a digital signal processor (RX8, Tucker Davis Technologies, Inc.). The loudness factor α was chosen as the minimum value that gives reliable learning during the conditioned feedback protocol, and was typically set at about 10.

$R(t)$ was constructed to give a large value when the pitch was within a specified range. This was achieved by using a bank of six bandpass filters arranged to detect the harmonic peaks in the squared audio signal of the targeted harmonic stack (Fig. S2A).

$$R(t) = \frac{F_1(t) + F_3(t) + F_5(t)}{\sum_{i=1}^{6} F_i(t)},$$

where the function $F_i(t)$ is the power measured in the squared song signal by i^{th} bandpass filter. The filter center-frequencies were spaced in an array. The filter shape (equi-ripple 300-450Hz full width) was chosen so that the feedback power fell off rapidly outside of a band of syllable pitches. In this way, a pitch threshold θ_p was defined, on one side of which the feedback power was large (greater than the birds own song) and on the other side of which the feedback power was small (Fig. 1B and S2C). This threshold pitch was set each morning before lights-on and remained constant for the rest of the day. The pitch threshold was set based on the last 50 non-TTX syllables of the previous day. Specifically, if the previous day was a TTX day, the threshold was set to average pitch of the last 50 syllables prior to inactivation. If the previous day was a vehicle day, the threshold was set to the average pitch of the last 50 syllables at the end of the day, prior to lights-out.

The value of $P_F(t)$ was used to modulate the amplitude of a broadband noise signal shaped to have the same spectral profile as the average spectrum of the experimental bird's song (Fig. S2B). Note that the feedback power was always scaled by the instantaneous song power, so that the bird could not reduce the relative feedback power by simply singing louder. All of the above filters were implemented as FIR filters on the digital signal processor. The filter widths were designed to be narrow in time so as to provide a very short delay (<4ms) between the singing and feedback signal. The loudness scale α was set such that the feedback was between 10 and 20dB louder than the target syllable.

Transient LMAN inactivation

After recovery from surgery, birds were connected to the electrical commutator and phosphate buffered saline (PBS) was infused into the drug reservoir every afternoon until the animals sang consistently following infusion. Drug infusion was carried out using the following procedure: Approximately 4 hours after the onset of singing, the bird was placed in a small foam restraint that left the head free and the microdialysis probe drug reservoir accessible. The caps were removed from the drug reservoirs. For infusion, the contents of the reservoir (vehicle) was extracted with the corner of a Kimwipe. A 1mL syringe containing the solution to be loaded (TTX or vehicle) was placed into the reservoir (Luer fitting). The solution was injected into the reservoir until approximately 50 μL of solution had flowed out through the outlet tube and the reservoir was filled. The syringe was removed and the caps were replaced. For washout, the contents of the reservoir (TTX or vehicle) were extracted with a Kimwipe, as described above, and 100 μL of PBS was injected through the outlet tube. The reservoir was then empied and filled again to ensure all TTX was rinsed from the reservoir and dialysis tubing. The reservoirs were left full of PBS, and the caps were replaced.

Typically, singing started within one hour of lights-on. The average number of syllables sung before drug infusion was 831 (5^{th}, 50^{th}, and 95^{th} percentile were 191, 691 and 1818, respectively). The amount of time between TTX infusion and singing averaged 78±72 minutes (s.d.), which was not significantly different than for vehicle infusion (62±48 minutes, s.d., p=0.25). On most TTX days, birds began to sing within an hour of infusion (n=19/34 infusions). The number of syllables sung during TTX infusion was not

significantly different than during vehicle infusion (TTX: mean 263±271 syllables, s.d.; vehicle: mean= 312±329 syllables, p=0.46). Reversals of pitch contingency (i.e. changing from moving up to moving down) were made every 4-8 days and were performed prior to lights-on on vehicle days.

To assess the extent to which pitch variability was reduced by TTX infusion, we calculated a reduction in variability based on the last 50 renditions of the target syllable prior to infusion and the first 50 renditions following infusion. For each set of 50 renditions, the mean time course of pitch was computed. This mean time course was subtracted from the individual pitch time courses within the set – resulting in a residual time course for each of the 50 syllables. Pitch variability was defined as the average of the standard deviations of these 50 residuals. Consistent with the fact that the standard deviation is calculated as variations around the mean value, residuals shown in Fig. S4A and B were demeaned (i.e. we subtract from each residual the mean pitch of that residual). The reduction in variability was calculated as the ratio of the post-infusion pitch variability to the pre-infusion pitch variability. These results were plotted as a function of TTX concentration (Fig. S4C). At a dose of 25μM, TTX infusion produced a saturating reduction in pitch variability, even for syllables produced within 30 minutes of infusion.

The spatial extent of inactivation produced by TTX infusion into LMAN was determined by electrophysiological recordings in anesthetized birds. First, we confirmed that medial MAN (MMAN) was not inactivated in our experiments. A microdialysis probe was stereotaxically placed in LMAN (1.75 mm LM), and an electrode (Carbostar, 1MΩ, Kation Scientific) was placed near the lateral edge of MMAN (0.6 mm and 0.7

mm LM, n=2 birds) to record the robust spontaneous bursting activity present throughout the nidopallium. Activity persisted in MMAN for the duration of the recording session, at least 1-1.5 hours following infusion of 25 μM TTX into the probe. The electrode was then moved laterally to record in steps of 0.2 mm. Complete loss of spontaneous activity was confirmed at lateral positions greater than 1.0 mm (within 0.75 mm of the probe).

Data analysis

The time courses of pitch and feedback power during the day (Fig. 1E and F) were computed as follows. The mean pitch and feedback power of every rendition of the target syllable was calculated for each day of training (n=80 days, n=5 birds). For each day, a smoothed trajectory of pitch and feedback power was calculated by averaging over blocks of 40 renditions (overlapping blocks, sliding by 10 renditions). Each smoothed trajectory was interpolated in 15 minutes steps. The initial morning value of pitch was subtracted from the trajectory for each day, and the pitch trajectory for down days was inverted. For the feedback power trajectory, the trajectory was normalized by the initial morning value of feedback power. The trajectories for all days were averaged together to produce the plots shown in Fig 1E and F. These plots include learning on TTX days, but only include singing prior to TTX infusion. The average rate of learning during the first four hours of the day (quoted in the results section) was computed from Fig 1E as the average pitch change at the four hour point, divided by four hours.

The distributions of daytime pitch changes include data from TTX and vehicle days (Fig. 1H). On vehicle days, the pitch change was between the first morning songs and the last evening song before lights-out. On TTX days, the pitch change was between the first morning songs and the last songs prior to TTX infusion. Overnight changes in pitch were quantified as the difference between the morning pitch and that of the final renditions of the preceding day. The overnight analysis included only nights following vehicle infusion (n=43 days).

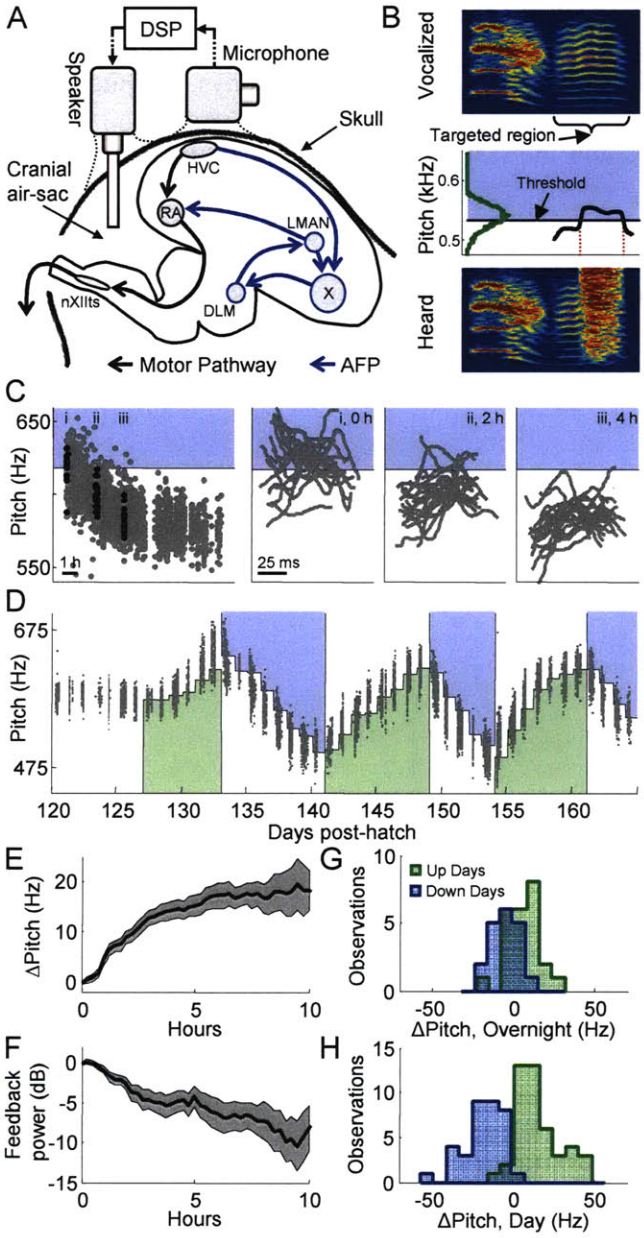

A
DSP
Microphone
Speaker
Skull
Cranial air-sac
HVC
RA
LMAN
X
nXIIts
DLM
Motor Pathway
AFP
B
Vocalized
Targeted region
Pitch (kHz)
0.6
0.5
Threshold
Heard
C
650
550
Pitch (Hz)
i
ii
iii
1 h
i, 0 h
ii, 2 h
iii, 4 h
25 ms
D
675
475
Pitch (Hz)
120
125
130
135
140
145
150
155
160
Days post-hatch
E
ΔPitch (Hz)
20
10
0
0
5
10
Hours
F
Feedback power (dB)
0
-5
-10
-15
0
5
10
Hours
G
Up Days
Down Days
Observations
10
5
0
-50
0
50
ΔPitch, Overnight (Hz)
H
Observations
15
10
5
0
-50
0
50
ΔPitch, Day (Hz)

Figure 1. Conditional feedback induces learning. (A) Schematic showing selected nuclei in the song bird brain and experimental apparatus to deliver conditional feedback. Vocal motor pathway (black arrows) and the anterior forebrain pathway (blue arrows, AFP), a basal ganglia-forebrain circuit necessary for learning. To induce learning, disruptive auditory feedback is played to the bird via a speaker implanted in the cranial airsac which is internally continuous with the eardrum. Feedback signals are computed with < 4ms delay by a digital signal processor (DSP), based on acoustic signals measured by a microphone on the head. HVC, proper name; RA, robust nucleus of the arcopallium; LMAN, lateral magnocellular nucleus of the nidopallium; X, Area X (proper name, homologous to the basal ganglia); DLM, dorsolateral nucleus of the medial thalamus; nXIIts, nucleus of the 12th nerve. (B) Schematic of conditional feedback protocol. Spectrogram of targeted syllable (top). A measure of pitch is computed continuously (middle, black curve). Whenever the pitch falls above a threshold (blue region) white noise is played to the bird (bottom). The threshold is positioned in the center of the pitch distribution of a region within the targeted syllable (green curve). (C) Left panel shows the average pitch (grey dots) of each rendition of the targeted harmonic stack sung over the course of the day, and the range of pitches for which feedback was played (blue region). The panels to the right show the pitch time course within the targeted harmonic stack for 20 consecutive renditions (black dots in left panel) at three time points during learning. D) Average pitch of each rendition of the targeted syllable (gray dots) for one experimental bird, plotted as a function of time (shading demarcates pitches that result in feedback; green, up days; blue, down days). (E) Average time course of pitch changes, relative to initial morning value, during a day of exposure to conditional feedback (down days inverted). Shaded area indicates s.e.m. F) Average time course of feedback noise power relative to initial morning value. (G) Histogram of the overnight change in pitch. (H) Histogram of learned pitch changes during each day of feedback.

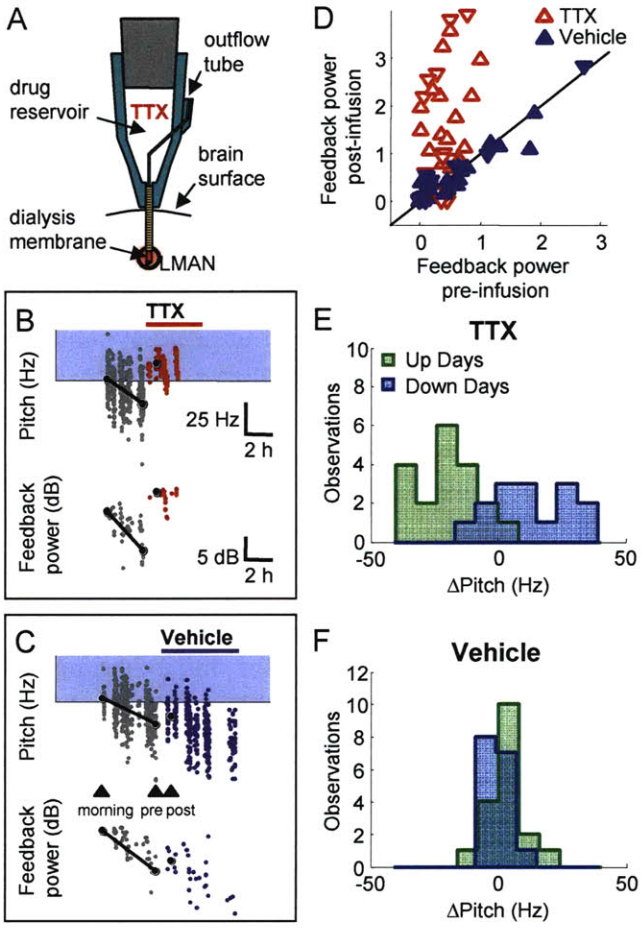

A
outflow tube
drug reservoir
TTX
brain surface
dialysis membrane
LMAN
B
TTX
Pitch (Hz)
25 Hz
2 h
Feedback power (dB)
5 dB
2 h
C
Vehicle
Pitch (Hz)
morning pre post
Feedback power (dB)
D
TTX
Vehicle
Feedback power post-infusion
Feedback power pre-infusion
E
TTX
Up Days
Down Days
Observations
ΔPitch (Hz)
F
Vehicle
Observations
ΔPitch (Hz)

Figure 2. LMAN inactivation reveals contribution of the AFP to vocal learning. (A) Reverse microdialysis probes are implanted bilaterally into LMAN, and tetrodotoxin (TTX) solution is held in a reservoir and diffuses through a porous membrane. (B) Average pitch (top panel) of each rendition of the targeted syllable during a day on which TTX was infused into LMAN (grey dots, pre-TTX; red dots, post-TTX). Also shown (bottom panel) is the feedback power played during the targeted syllable (dots represent averages of 10 sequential renditions). Black dots indicate the mean pitch in the morning, pre-infusion, and post-infusion. (C) Same as (B) except vehicle was infused (purple dots). Note that during vehicle infusions the pitch of the targeted syllables continued to exhibit learning in the instructed direction (1.26±0.66Hz/hr, $p<0.03$, two-tailed t-test), whereas learning stopped during TTX infusions (-0.50±0.62 Hz/hr, $p>0.7$, two-tailed t-test). (D) Feedback noise power, pre-infusion versus post-infusion (TTX, red hollow symbols; vehicle, purple filled symbols). Black line indicates unity slope. (E) Histogram of the effect of TTX infusion on pitch (post- minus pre- infusion). Note the regression of pitch opposite the ongoing direction of learning. (F) Histogram of the effect of vehicle infusion on pitch.

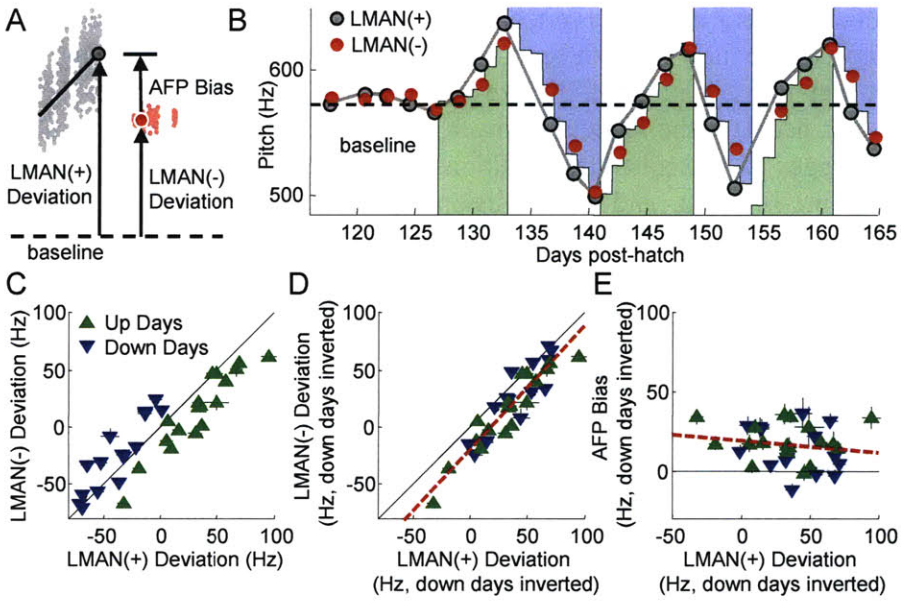

A
AFP Bias
LMAN(+)
Deviation
LMAN(-)
Deviation
baseline
B
LMAN(+)
LMAN(-)
Pitch (Hz)
600
500
baseline
120 125 130 135 140 145 150 155 160 165
Days post-hatch
C
Up Days
Down Days
LMAN(-) Deviation (Hz)
100
50
0
-50
-50 0 50 100
LMAN(+) Deviation (Hz)
D
LMAN(-) Deviation
(Hz, down days inverted)
100
50
0
-50
-50 0 50 100
LMAN(+) Deviation
(Hz, down days inverted)
E
AFP Bias
(Hz, down days inverted)
100
50
0
-50 0 50 100
LMAN(+) Deviation
(Hz, down days inverted)

Figure 3. The motor pathway contributes to accumulated changes in pitch. (*A*) Schematic showing the deviation of the preinactivation pitch [LMAN(+), large gray dot] and postinactivation pitch [LMAN(-), large red dot] from the average syllable pitch before the first exposure to conditional feedback (baseline pitch, dashed line). (*B*) Time series of LMAN(+) and LMAN(-) pitch for all experimental days in 1 bird. (C) Scatter plot of the deviation of LMAN(-) pitch and LMAN(+) pitch from the baseline pitch for all inactivations (error bars indicate 3 SE). (D) Same as (C), but data from up and down days are combined by inverting the sign of the deviation for down days (linear regression, red dashed line). (E) Scatter plot of AFP bias versus the deviation of LMAN(+) pitch from baseline (data from down days are inverted; linear regression, red dashed line; slope not significantly different from zero).

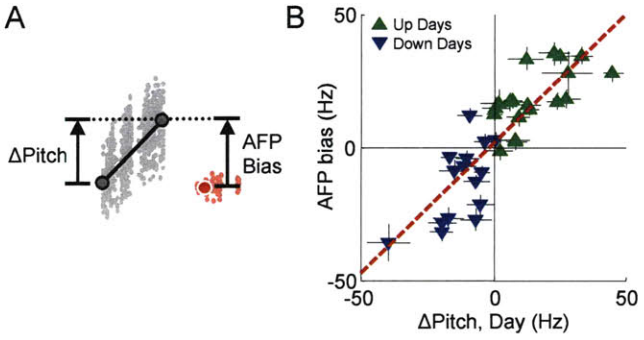

A
ΔPitch
AFP
Bias
B
Up Days
Down Days
AFP bias (Hz)
50
0
-50
-50
0
50
ΔPitch, Day (Hz)

Figure 4. AFP bias is correlated with the amount of learning in the morning prior to inactivation. (A) Schematic illustrating the measurement of pitch learned during the day and AFP bias. (B) Scatter plot of the AFP bias versus amount of learning in the morning prior to infusion reveals strong correlation (linear regression, red dashed line).

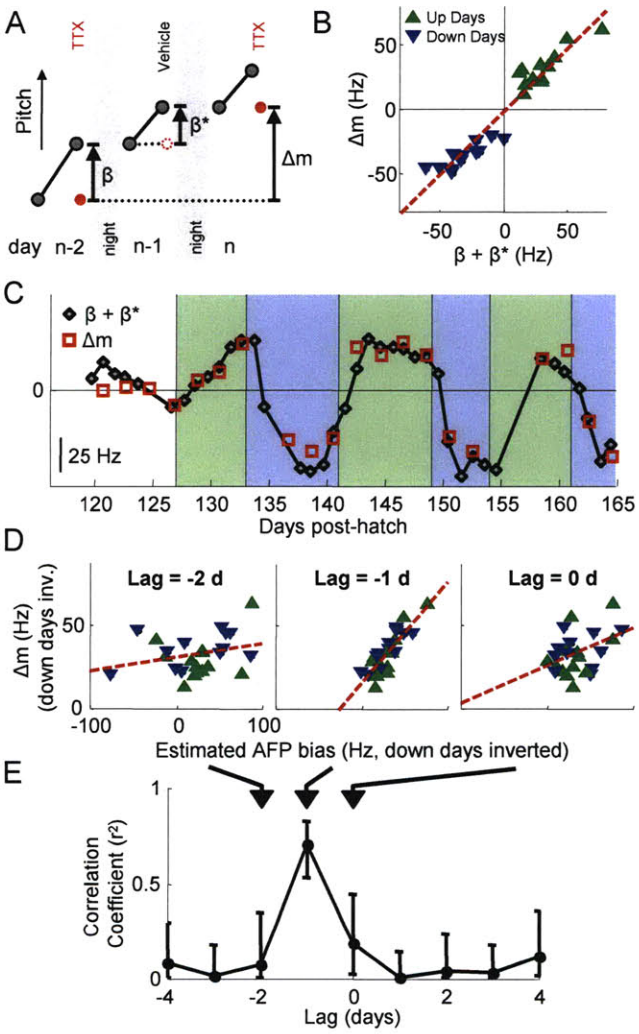
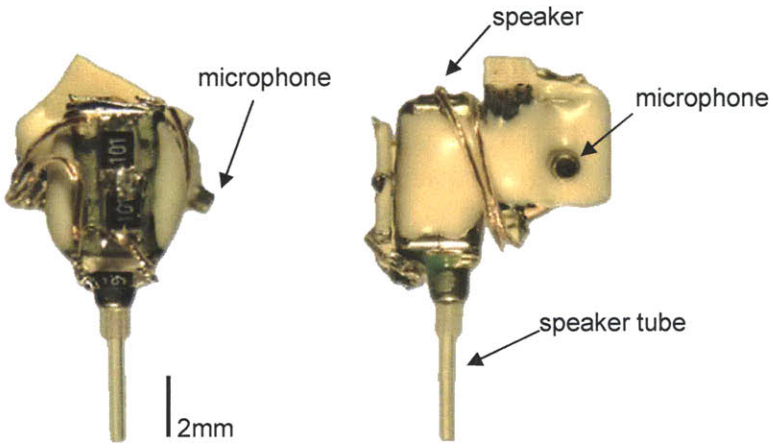

A
TTX
Vehicle
TTX
Pitch
β*
β
Δm
day n-2 night n-1 night n
B
Up Days
Down Days
Δm (Hz)
50
0
-50
-50 0 50
β + β* (Hz)
C
β + β*
Δm
0
25 Hz
120 125 130 135 140 145 150 155 160 165
Days post-hatch
D
Lag = -2 d
Lag = -1 d
Lag = 0 d
Δm (Hz)
(down days inv.)
50
0
-100 0 100
Estimated AFP bias (Hz, down days inverted)
E
Correlation
Coefficient (r²)
1
0.5
0
-4 -2 0 2 4
Lag (days)

Figure 5. AFP bias is predictive of subsequent plasticity in the motor pathway. (A) Motor plasticity was assessed as the difference (Δm) in LMAN(-) pitch between successive inactivations (red dots), carried out every other day. Gray dots represent morning and pre-inactivation syllable pitch. Motor plasticity was correlated to the estimated total AFP bias over a corresponding two day interval. The total was computed as the sum ($\beta + \beta^*$) of the AFP bias on TTX days (β) plus the AFP bias on vehicle days (β^*) – the latter estimated as the amount of learning that occurred during the day (see Fig. 4). (B) Scatter plot of motor plasticity (Δm) and estimated two-day sum of AFP bias ($\beta + \beta^*$; linear regression, red dashed line; slope = 0.99±0.06, r2 = 0.93). (C) Time series of Δm (red squares) and a two-day running sum of estimated AFP bias one day earlier, as shown in panel A (black diamonds). (D) Scatter plots showing the correlation between Δm versus the estimated two-day sum of AFP bias at lags of -2, -1, and 0 days (linear regression, red dashed line; slopes = 0.08±0.06, 0.61±0.08, 0.23±0.10 respectively; up and down days combined by inverting down days; see Fig. S7). Panels A-C correspond to a lag of -1 day. (E) Correlation coefficients as a function of time lag (days). Errors bars are 95% confidence intervals.

A


microphone
speaker
microphone
speaker tube
2mm

B

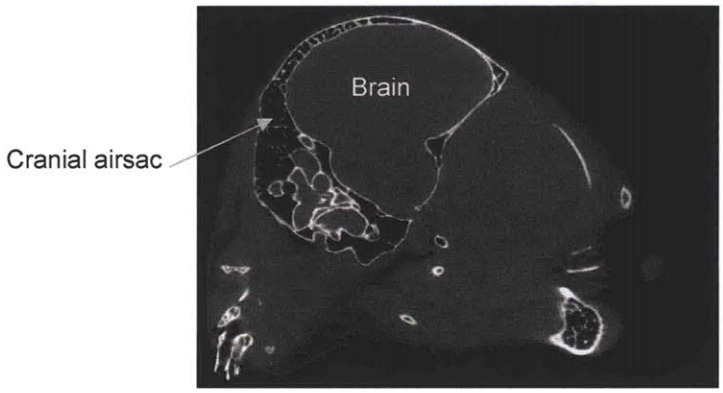

Brain
Cranial airsac

Figure S1. Disruptive auditory feedback system. (A) Photographs of speaker implant. A hearing-aid speaker generated broadband noise to distort the auditory feedback of the bird during singing. The sound was delivered through a speaker tube into the cranial airsac. Song was recorded using a miniature microphone attached to the side of the implant. (B) The cranial airsac as seen in a parasagittal CT-section through the middle ear. This airsac is continuous with the inner surface of both eardrums.

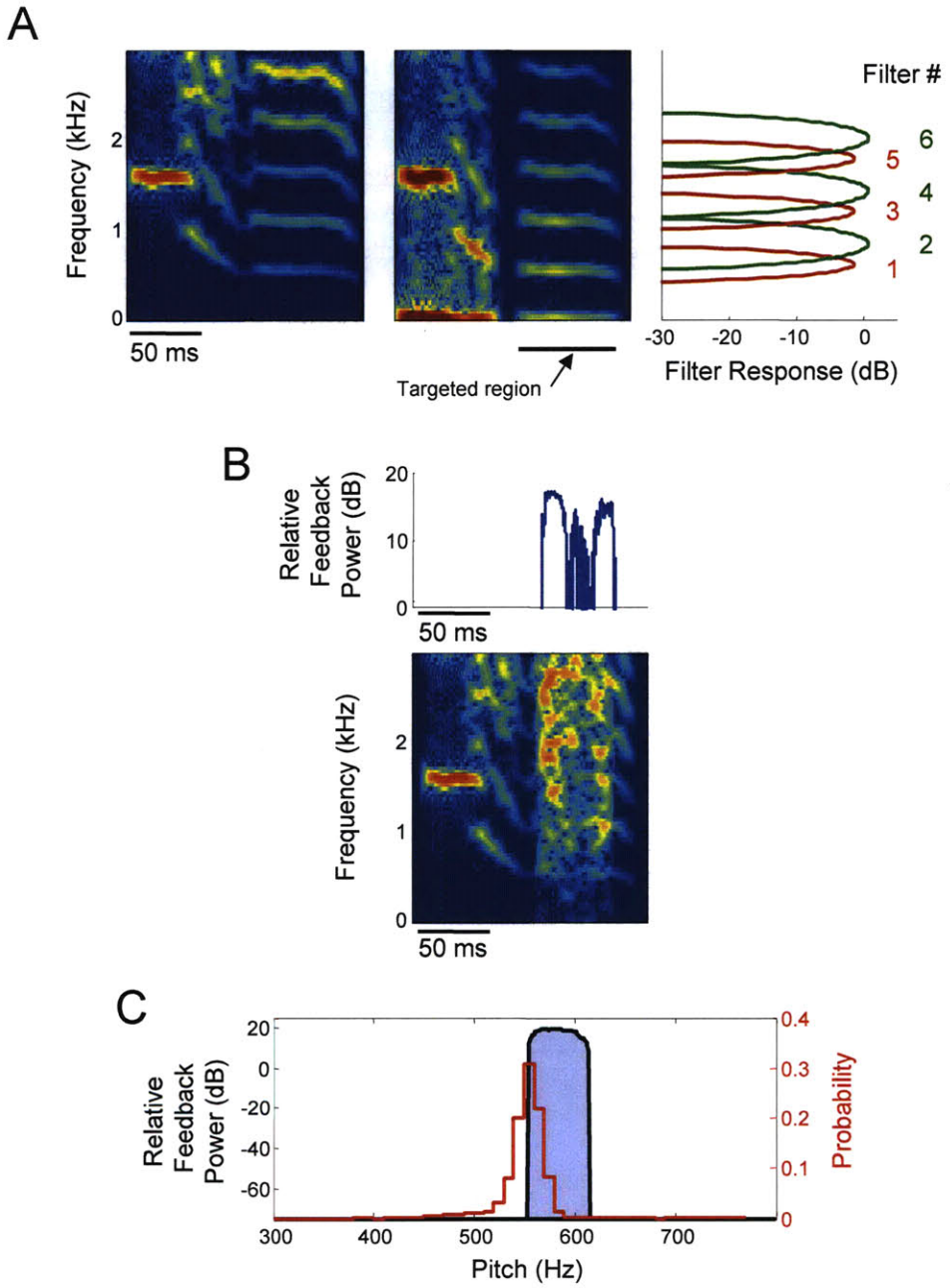

A
Frequency (kHz)
2
1
0
50 ms
Targeted region
Filter #
6
5
4
3
2
1
-30
-20
-10
0
Filter Response (dB)
B
Relative Feedback Power (dB)
20
10
0
50 ms
Frequency (kHz)
2
1
0
50 ms
C
Relative Feedback Power (dB)
20
0
-20
-40
-60
0.4
0.3
0.2
0.1
0
Probability
300
400
500
600
700
Pitch (Hz)

Figure S2. Method for generating disruptive auditory feedback conditional on pitch. (A) Spectrogram of one song syllable (left panel) and the associated spectrogram of the squared audio signal (middle panel). The latter is a better measure for pitch because it is less affected by timbre (i.e. missing harmonics). A particular pitch range is targeted for feedback using a bank of 6 bandpass filters – three arranged to detect in-band power and three arranged to detect out-of-band power (right panel; red, in-band; green, out-band). (B) The feedback power (relative to song power) is proportional to the sum of the three in-band filters, normalized by the sum of all six filters (top panel). Also shown is the distorted auditory feedback signal superimposed over the syllable spectrogram (with correct amplitude scaling, bottom panel). (C) Relative feedback power as a function of pitch (black trace) for the filter settings used in panels A and B. Also shown is the distribution of pitches for the targeted syllable of the syllable shown above (red trace). Note that the edge of the pitch detection region (shaded) is set approximately to the middle of the distribution of pitches of the targeted syllable.

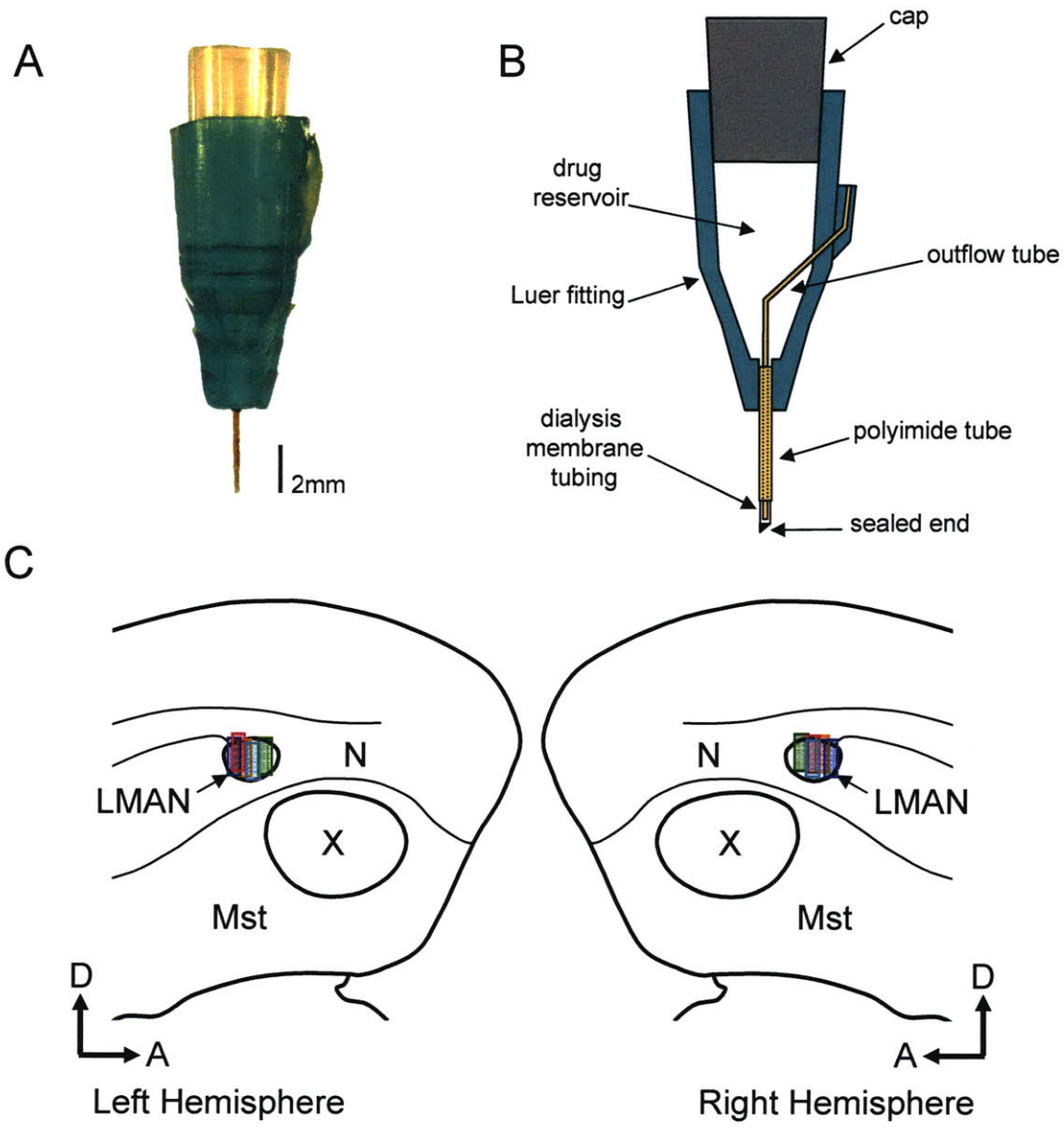

A
2mm
B
cap
drug reservoir
outflow tube
Luer fitting
dialysis membrane tubing
polyimide tube
sealed end
C
N
LMAN
X
Mst
D
A
Left Hemisphere
N
LMAN
X
Mst
D
A
Right Hemisphere
aa275
aa266
aa295
aa291
aa259

Figure S3. Untethered reverse microdialysis for inactivation of LMAN. (A) Photograph of microdialysis probe. (B) Schematic drawing of microdialysis probe showing drug reservoir, dialysis tubing, and outflow tube. The outflow tube is inserted concentrically to the bottom of the sealed end of the dialysis tubing, allowing fluid to be rapidly flowed in and washed out of the dialysis tubing. (C) Histologically confirmed location of bilateral microdialysis probes in LMAN in all 5 experimental birds in the parasagital plane. All probes were also within 300μm of the center of LMAN along the medial-lateral axis. (bird numbers listed at bottom; Abbreviations: X, Area X; N, nidopallium; Mst, medial striatum; LMAN, lateral magnocellular nucleus of the nidopallium; D, dorsal; A, anterior).

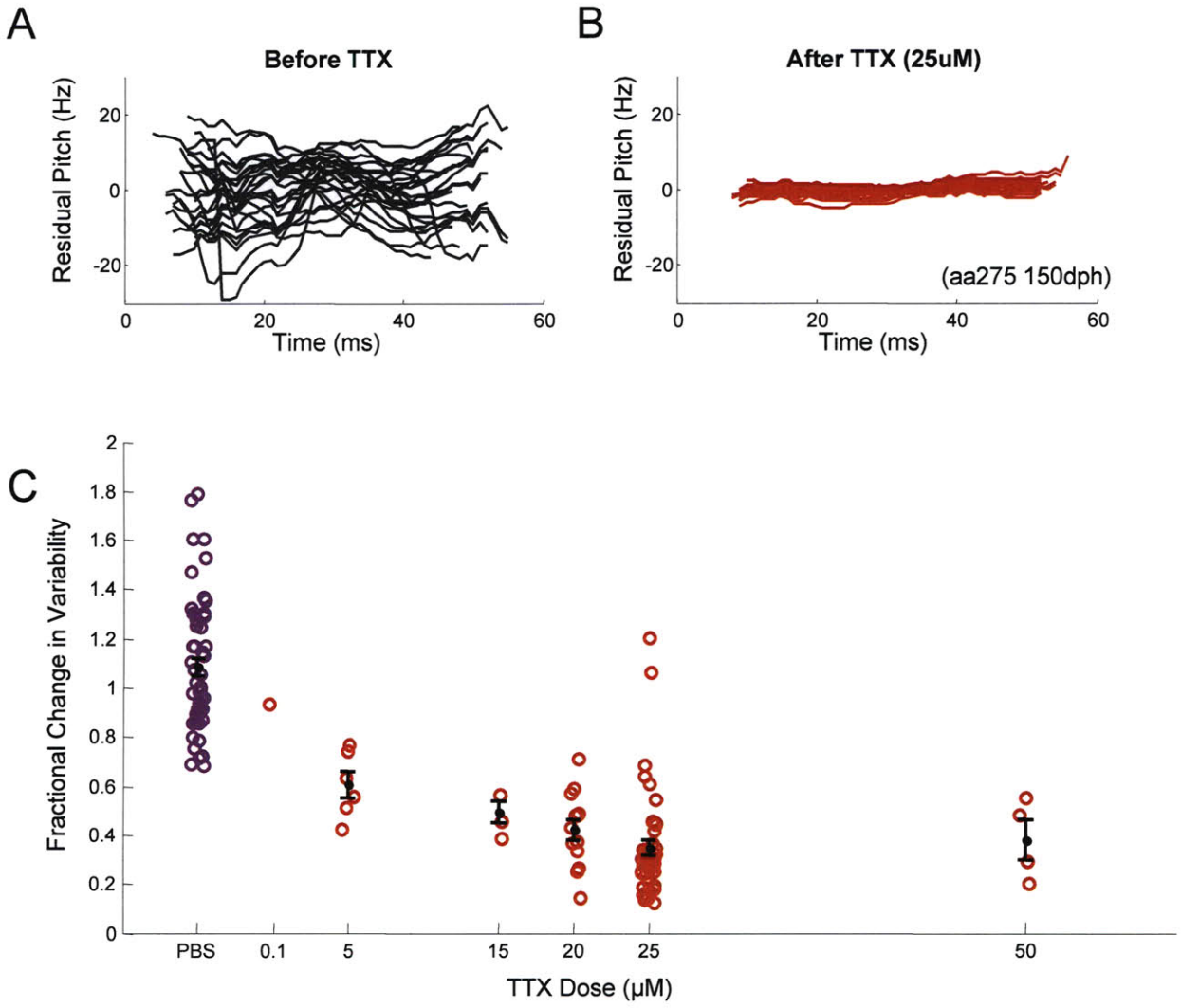

A
Before TTX
Residual Pitch (Hz)
20
0
-20
0
20
40
60
Time (ms)
B
After TTX (25uM)
Residual Pitch (Hz)
20
0
-20
(aa275 150dph)
0
20
40
60
Time (ms)
C
Fractional Change in Variability
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
PBS
0.1
5
15
20
25
50
TTX Dose (μM)

Figure S4. Effect of tetrodotoxin (TTX) in LMAN on variability of pitch. (A) Time course of residual pitch for the last 25 renditions of the targeted syllable prior to infusion (bird #aa275, 150 dph). (B) Time course of residual pitch for same targeted syllable after infusion of 25μM TTX into reverse microdialysis probe (first 25 renditions post-infusion). Pitch variability following infusion was 18% of the pre-infusion variability (see Supplementary Methods). (C) Dose-response curve for reduction in pitch variability for 6 concentrations of TTX, compared to vehicle infusion (PBS). A concentration of 25μM produced a saturating reduction in pitch variability, and did not affect the rate of singing. Black symbols indicate the mean and standard error for each concentration.

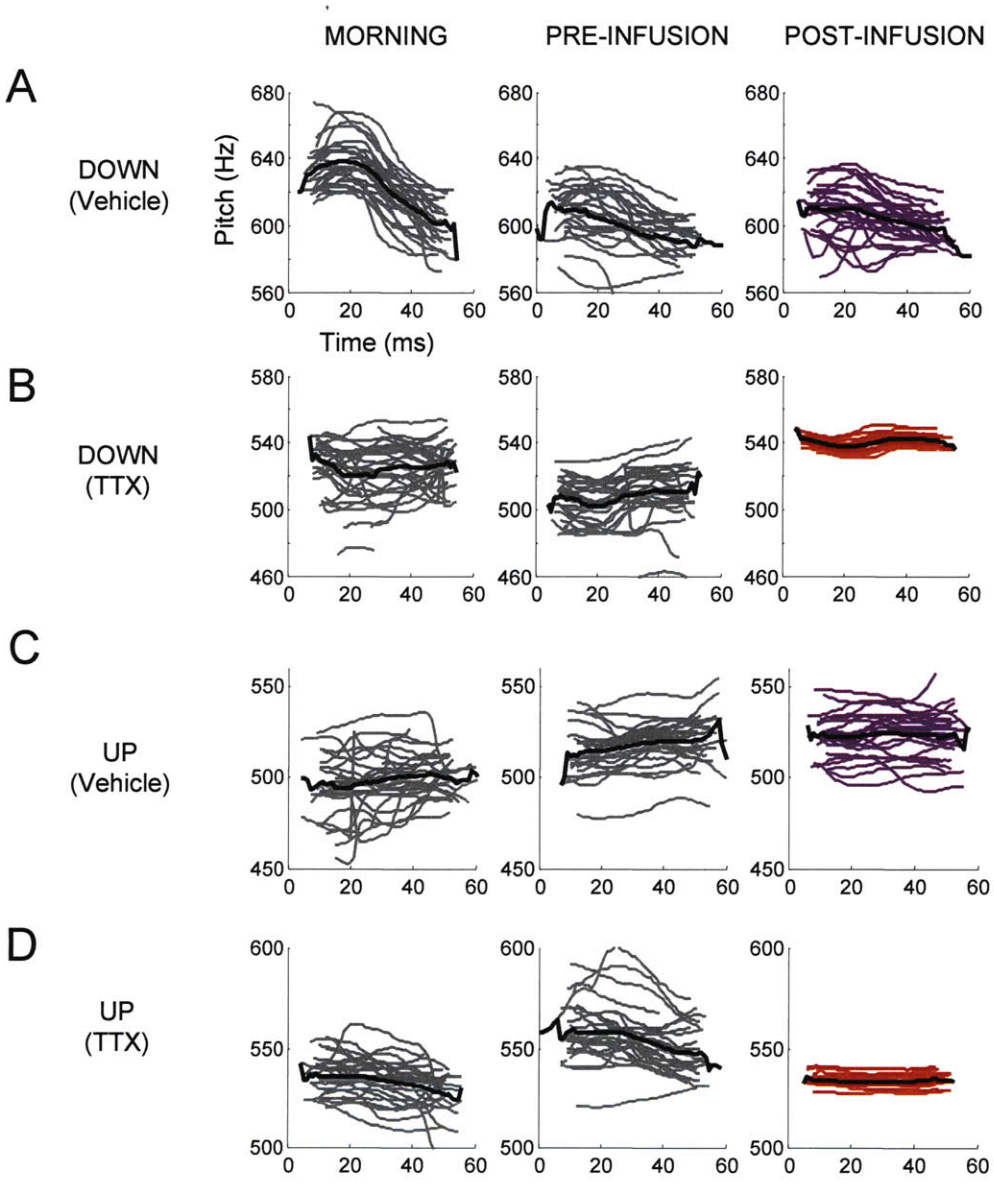

MORNING
PRE-INFUSION
POST-INFUSION
A
DOWN
(Vehicle)
B
DOWN
(TTX)
C
UP
(Vehicle)
D
UP
(TTX)
Pitch (Hz)
Time (ms)

Figure S5. Examples of pitch time courses on four experimental days. (A-D) Each curve reflects the time course of pitch within one rendition of the targeted syllable. Shown are the first 25 renditions of the day (morning, left column), followed by 25 syllable renditions after several hours of learning under conditional feedback (immediately prior to drug infusion, middle column). Note that during the time between awaking (morning) and manipulation (pre-infusion), the distribution of pitches has shifted down for days on which the pitch is being pushed down (DOWN) and has shifted up for days on which the pitch was pushed up (UP). Also shown are pitch traces for the first 25 syllable renditions after infusion (post-infusion, right column; red lines, TTX, B and D; purple lines, vehicle, A and C). Note the dramatic reduction of pitch variability during TTX inactivation, and the regression of average pitch back toward the morning value. Mean time course shown in black. Panels A and B show the time courses from the experimental days shown in Fig. 2C and Fig. 2B (bird #aa275, 149dph and 152dph). Panels C and D show two additional experimental days (bird #aa275, 141dph and 142dph).

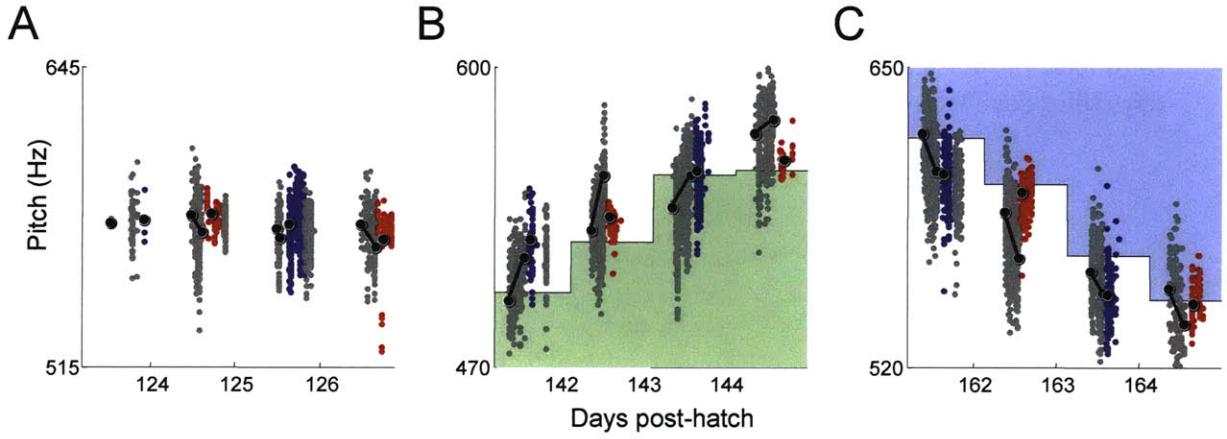

A
B
C
645
515
Pitch (Hz)
124
125
126
600
470
142
143
144
Days post-hatch
650
520
162
163
164

Figure S6. Sequential experimental days. (A) Four sequential days of drug infusion during the baseline period, before application of conditional feedback. TTX (red) and vehicle (purple) infused on alternate days. Black dots indicate average pitch in the morning, pre-infusion, and post-infusion periods. (B,C) Four sequential days of drug infusion during experimental days when conditional feedback was used to move pitch up (B) and down (C).

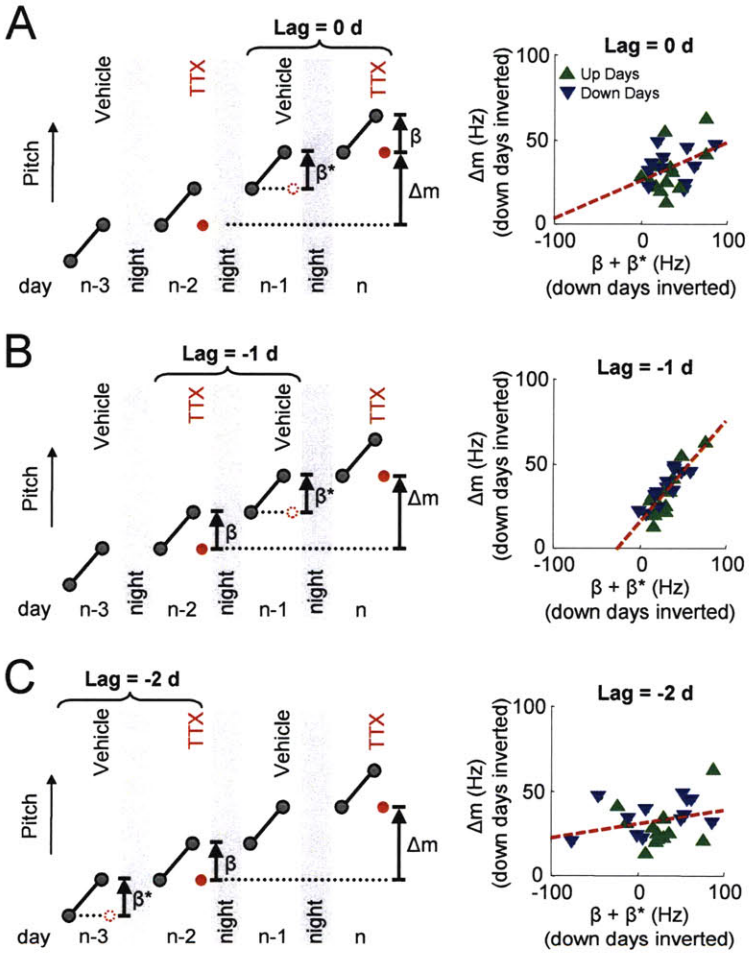

A
Lag = 0 d
Vehicle
TTX
Vehicle
TTX
Pitch
β
β*
Δm
day
n-3
night
n-2
night
n-1
night
n
Lag = 0 d
Up Days
Down Days
Δm (Hz)
(down days inverted)
100
50
0
-100
0
100
β + β* (Hz)
(down days inverted)
B
Lag = -1 d
Vehicle
TTX
Vehicle
TTX
Pitch
β
β*
Δm
day
n-3
night
n-2
night
n-1
night
n
Lag = -1 d
Δm (Hz)
(down days inverted)
100
50
0
-100
0
100
β + β* (Hz)
(down days inverted)
C
Lag = -2 d
Vehicle
TTX
Vehicle
TTX
Pitch
β
β*
Δm
day
n-3
n-2
night
n-1
night
n
Lag = -2 d
Δm (Hz)
(down days inverted)
100
50
0
-100
0
100
β + β* (Hz)
(down days inverted)

Figure S7. Detailed illustration of the how the correlation between variations in motor pathway plasticity and estimated AFP bias was computed at different lags for Figure 5. (A,B,C) The left portion of each panel is a schematic showing the quantities used to calculate the change in LMAN(-) pitch (Δm) and the estimated AFP bias ($\beta + \beta^*$) at different lags (0, -1, and -2 days, respectively). Note the quantities used to estimate AFP bias shift in time according the lag value. The right portion of each panel shows the scatter plot of Δm versus $\beta + \beta^*$ at the corresponding lag.

Chapter 5: Discussion

The neurobiology of vocal learning in the zebra finch: insights into the function of basal ganglia-forebrain circuitry

To synthesize the experiments described in the preceding chapters, it is helpful to assemble the results into a simple model of how song learning proceeds. This model provides an explanation at a systems level of how the two major neural circuits in the song system (Fig. 1A) contribute differentially to vocal learning. The key assertions of the model are: first, the AFP, an avian homologue of basal ganglia-thalamocortical circuitry, actively drives exploratory singing that is necessary for reinforcement learning; and second, the AFP uses the evaluation of auditory feedback to generate a corrective premotor drive which subsequently serves to guide plasticity in the motor pathway.

This chapter introduces the model and then presents supporting evidence, alternative interpretations, and possible lines of research to test and extend the model. Finally the chapter assesses the significance of the model's key assertions for understanding the function of the basal ganglia in motor learning. In particular this model further supports and significantly refines the view that basal ganglia-forebrain circuitry is involved in reinforcement learning. The two essential components of reinforcement learning are variable behavior and the evaluation of behavioral outcomes. The model suggests a specific function of basal ganglia-forebrain circuitry with regard to both of these components: that these circuits act as a dedicated source of behavioral variability, and that they are capable of using the evaluation of past action to generate temporally precise premotor drive that incrementally improves performance of a motor skill.

Synthesis of results: a simple model of song learning

Song learning, or imitation of any sort, can be thought of as an optimization problem in which similarity with a target is maximized (Billard, Epars et al. 2004). There exists a large high-dimensional space of zebra finch songs, and the bird attempts to navigate this space to find the point at which the similarity between the bird's own song and the template song – the song the bird is attempting to imitate – is maximal.[1]

For simplicity, consider the space of all songs that a bird can sing as having only two dimensions -- a simple Cartesian coordinate system. In reality, of course, the space of all songs has many more dimensions, but for the purposes of the model two dimensions will suffice.

The motor pathway encodes a stereotyped song, even before song crystallization

The motor pathway, even in the juvenile bird, is capable of producing a stereotyped song. The experiments described in chapter two established that songs produced by juvenile birds, when LMAN [2] is transiently inactivated with local injection of either tetrodotoxin (TTX) or muscimol, are as similar from rendition to rendition as adult undirected songs (songs not directed toward a female bird). Consequently, the song encoded by the motor pathway, even in the learning bird, can be represented by just a single point in the model's simplified space of songs (Fig. 1B).

[1] The bird does not necessarily discover the point of maximal similarity. More likely the bird arrives at a satisfactory local maximum.

[2] It is important to note that the projection from LMAN (part of the AFP) to the RA (robust nucleus of the arcopallium, part of the motor pathway) is the primary output of the AFP. It is this projection that allows the AFP to influence the motor pathway.

The AFP drives variability in juvenile song

A corollary of this finding is that the AFP is introducing variability into the juvenile song such that it is rendered differently every time the bird sings. Recordings from individual RA-projecting neurons in LMAN show that their spiking activity during singing is highly variable in juvenile birds singing plastic song (chapter 2). In fact, the activity of these neurons has a premotor correlation with the highly unstructured and variable syllables produced by birds singing subsong (chapter 3). Additionally, infusing AP-V into RA, which blocks glutamatergic input from the AFP to the motor pathway, causes a reduction of variability nearly equivalent to inactivating LMAN with tetrodotoxin (chapter 2). These results suggest that the variability prominent in juvenile song is produced by variable excitatory glutamatergic drive from the AFP to the motor pathway. This variability can be represented in the two-dimension song space as a scattering of points around the motor pathway, each point representing one particular rendition of song (Fig. 1B). Evidence suggests that in birds whose song is stable, i.e. adult birds, the variability introduced by LMAN is unbiased (Kao, 2005).

Variability enables reinforcement learning

Reinforcement learning proceeds by reinforcing behaviors that result in better outcomes, but this process stalls if the same outcome occurs over and over. In other words, variability in behavior is critical to a reinforcement learning process. Variability in song could have originated from immature circuitry in the motor pathway (Kittelberger and Mooney 1999) – where the adult program for singing is eventually stored (Nottebohm, Stokes et al. 1976; Long and Fee 2008). Instead, it appears to be actively

added to a relatively stereotyped motor pathway program by a dedicated neural circuit, the AFP. This explicit addition of variability suggests that it serves an important function, perhaps as a key ingredient in a reinforcement learning process.

From the reinforcement learning perspective, AFP-generated variability permits learning by enabling the discovery of motor outcomes that improve the match between the bird's own song and the template song (Sutton and Barto 1998; Fiete, Fee et al. 2007). The ability of conditional auditory feedback paradigms to induce learning supports this view, (Tumer and Brainard 2007)(chapter 4). These paradigms make disruptive feedback contingent on the natural variability in the song. The fact that these paradigms induce learning indicates that the brain is capable of harnessing this variability for learning. Viewed in the context of a song sitting in a high-dimensional space, variation in each rendition allows the bird to sample the space around the current motor program and determine in which direction the song should move – i.e. determine the gradient of song similarity around the current motor program. If the bird is already successfully imitating the template, then no direction of movement will result in improvement. If, however, the bird is not yet imitating well, the bird will discover the direction in which imitation can be improved (Fig. 1C).

The AFP provides corrective premotor bias

The model thus far provides a possible explanation for how the song system discovers which slight modifications to the song will improve imitation. But how might learning proceed from here? Experiments testing the function of the AFP during

conditional auditory feedback provide a strong indication. Inactivation of LMAN while the bird is learning to change the pitch of a syllable – in order to reduce the amount of disruptive auditory feedback the bird hears– causes an immediate reversion of pitch in a direction opposite that of ongoing learning (chapter 4). This suggests that while the bird is learning the AFP contributes more than simple unbiased variability. Rather, the AFP provides an adaptive signal which is contributing to the changes in pitch that reduce the amount of disruptive auditory feedback the bird hears. This adaptive signal can be thought of as a bias in the variability introduced by the AFP (AFP bias). In song space, the scatter of points produced by variability becomes shifted in the direction of the similarity gradient (Fig. 1D). The size of this bias is limited, and it is proportional to the amount of the learning that has taken place most recently (within the last four hours, chapter 4).

AFP bias guides plasticity in the motor pathway

The adult program for singing is eventually encoded in the motor pathway (Nottebohm, Stokes et al. 1976; Long and Fee 2008); therefore, song learning must eventually be expressed as plasticity in this pathway. The data presented in chapter 4 suggest that AFP bias might guide plasticity in the motor pathway. The size of the AFP bias was strongly predictive of the amount of plasticity in the motor pathway during the subsequent day. In addition, the average magnitude of the AFP bias was not significantly different from the amount of learning that occurred in the motor pathway on a day-to-day basis. In terms of song learning, these findings suggest that, initially, improvements to the

song are driven by the AFP, but on the time scale of about a day these improvements are transferred into the motor pathway (Fig. E and F).

Model summary

The following picture emerges from the research results taken together: variability driven by AFP allows motor space to be explored; on a time scale of hours the AFP discovers which modifications improve the song and biases motor output in that direction; and on a time scale of about a day these improvements become encoded in the motor pathway. These three mechanisms proceed in parallel until the bird produces a satisfactory imitation of the template song.

Discussion of the model: supporting evidence and experimental tests

The data in support of this model are compelling, but there are, of course, caveats and alternative interpretations. In this section, these alternate interpretations are discussed in relation to other work in the field, preliminary data, and possible experimental tests.

AFP drives song variability

Prior work has shown that permanent lesions of LMAN in juvenile birds cause a dramatic reduction of spectral and sequence variability (Bottjer, Miesner et al. 1984; Scharff and Nottebohm 1991). As discussed in chapter 1, this variability was previously hypothesized to be the result of synaptic reorganization in RA due to a loss of neurotrophic factors (Kittelberger and Mooney 1999). Chapters 2 and 3 suggest the alternate view that it is loss of excitatory glutamatergic synaptic input from LMAN that

reduces variability. This conclusion is further supported by other recent findings. Songs directed at females (directed song) contain less variability than songs produced by isolated adults (undirected song) (Kao 2005). This adult variability appears to be LMAN-dependent: spiking patterns in LMAN are more variable during undirected song (Hessler and Doupe 1999); and lesions of LMAN eliminate the increased variability of undirected song (Kao, Doupe et al. 2005; Kao and Brainard 2006). Finally, microstimulation in LMAN causes a transient perturbation of learned motor output, as would be expected if LMAN provided premotor drive (juveniles see Fig. 2; adults see Kao et. al., 2005).

To draw a direct mechanistic link between spiking in LMAN and variability in song, one would want to show a correlation between individual spikes in LMAN and the modulation of a song parameter. Single-unit recordings in RA in juvenile birds reveal that spiking patterns in RA become less variable when LMAN is transiently inactivated (unpublished data). In addition, variations in RA spiking patterns have been correlated with variations in spectral features of the song (Sober, Wohlgemuth et al. 2008). These two results strongly support a direct link between the spiking patterns in LMAN and variations in song. While it is conceivable that rewiring due to loss of trophic factors in RA also causes reduced variability, postulating such a mechanism appears unnecessary.

Is AFP bias a corrective premotor drive?

The proposed model of song learning suggests that when there is a direction in which modifying the song will improve the match between auditory feedback and the template song, the variability produced by the AFP becomes biased so as to provide

corrective premotor drive. This aspect of the model is supported by experiments described in chapter 4 in which inactivation of LMAN during feedback-induced vocal learning is found to cause a regression of on-going learning. This finding clearly demonstrates, for the first time, an AFP-dependent contribution to vocal learning. But is this contribution a premotor signal mediated by fast synaptic input from LMAN to RA?

The view that the AFP's contribution to biasing vocal output is premotor is a natural extension of the AFP's premotor role in producing variability. The possibility that the same glutamatergic input that modulates song parameters variably across renditions might be capable of modulating the parameters in a consistent way seems a minimal logical leap. This leap is supported by the finding that time-locked microstimulation of LMAN can induce brief and consistent changes in a song parameter (Kao, Doupe et al. 2005). Time-locked microstimulation presumably causes LMAN activity to be correlated with the motif; and such correlations have been observed under natural conditions (chapter 2) (Hessler and Doupe 1999; Kao, Doupe et al. 2005; Kao and Brainard 2006). These findings suggest that LMAN neurons have all the attributes necessary to bias activity in RA neurons during singing.

Is AFP bias mediated by NMDA receptors?

Nonetheless, there are alternative means by which the AFP could bias motor output. For instance, LMAN could release a trophic factor which is necessary for the expression of premotor bias encoded elsewhere. Establishing that AFP bias is dependent on glutamatergic input from LMAN to RA would obviate this possibility. By infusing

AP-V in RA, analogous to methods used in chapter 2, glutamatergic input from LMAN to RA could be blocked during feedback-induced learning. If this technique resulted in a regression of learning, it would indicate that the AFP's contribution to learning is mediated by NMDA receptors in the LMAN to RA synapse, not by trophic factors.

Finding a neural correlate of AFP bias

It would be informative to find a neural correlate of AFP bias using electrophysiology. Previous work has shown that the activity patterns of LMAN neurons do not show a real-time response to disruptive auditory feedback (Leonardo 2004). This, at first blush, may seem inconsistent with a premotor hypothesis. However, corrective premotor drive is necessarily a complex function of auditory feedback involving correlating past performance with neural activity. Given this complex relationship, one may not expect LMAN activity to show real-time responses to auditory feedback.

The logical place to look for a neural correlate of AFP bias is LMAN, since it the nucleus which connects the AFP to the motor pathway. The experiment would involve recording an LMAN neuron while using conditional auditory feedback to induce learned changes in song. If AFP bias were encoded in the spiking patterns of LMAN neurons – rather than in the strength of the synapses connecting LMAN to RA or in AFP-dependant motor pathway plasticity – then motif-aligned patterns of activity in LMAN projection neurons would change as the bird learned. Demonstrating such a change in a subset of LMAN neurons would strongly suggest that AFP bias reflects fast synaptic premotor drive from LMAN.

Does AFP bias occur in natural song learning?

A second question regarding AFP bias is does it occurs during natural song imitation, or is it an artifact of the conditional auditory feedback paradigm? Two arguments counter the idea that it is an artifact. First, conditional auditory feedback is designed to realistically simulate natural auditory feedback. The volume levels are similar to the bird's own song; the white noise used as feedback is filtered to have the same average spectrum as the bird's own vocalizations; and the feedback played into the skull is independent of position and orientation.

Second, the neural circuits involved in conditional auditory feedback-induced learning are the same circuits that have been implicated in natural song learning (Bottjer, Miesner et al. 1984; Brainard and Doupe 2000). One question often posed is whether AFP bias could be observed in a paradigm that used a mild foot-shock in place of disruptive auditory feedback. However, this question does not directly address the fundamental concern. If a conditional foot-shock experiment resulted in learning, and also revealed an AFP contribution to learning, then it would suggest that the neural circuits that process auditory feedback converge with the circuits that process other types of feedback before they are used for motor learning. This would not be particularly surprising since the bird might want to optimize its song in response to both auditory feedback as well as other non-auditory cues (including perhaps social cues during performance).

A direct test of whether AFP bias occurs during natural song imitation would involve inactivating LMAN in young juvenile birds that are still acquiring their songs. Inactivations of LMAN would be carried out in the afternoon in birds singing plastic song. Various spectral features would be computed on all the syllables produced by the bird. Natural learning would occasionally cause these features to change during the course of the day prior to inactivation. If AFP bias is a natural phenomenon, then inactivation will cause these song features to revert toward their morning values.

Does AFP bias guide plasticity in the motor pathway?

A critical component of the model is that AFP bias guides motor pathway plasticity. This idea is supported by the strong correlation between the magnitude of AFP bias and the amount of plasticity observed in the motor pathway on the subsequent day (chapter 4). In fact, the magnitudes of these two values were not significantly different. This correlation, of course, does not prove a mechanistic relationship between AFP bias and motor pathway plasticity, but the idea is supported by the fact that LMAN is necessary for plasticity (Williams and Mehta 1999; Brainard and Doupe 2000). Further experimentation will be required to understand if and how AFP bias and motor pathway plasticity are mechanistically related.

One weakness of the correlation presented in chapter 4 is that the values are only measured in a particular experimental condition at a single time point during the day. A clearer understanding of the relationship between AFP bias and motor pathway plasticity could be gained by simply sampling more frequently -- for instance, by probing motor

pathway plasticity and AFP bias every hour using a rapid, minutes-timescale, inactivation technique (TTX, used in chapter 2, lasts for many hours). Hourly inactivations could be performed as the contingency for auditory feedback was modified to reverse the direction of learning. As the bird switches from learning to sing a higher pitch to learning to sing a lower pitch, how quickly does the AFP bias change? How long before plasticity begins to occur in the motor pathway? And how do these values relate to each other?

Although there is significant insight to be gained from such detailed probing of the relationship between AFP bias and motor pathway plasticity, this sort of experimentation cannot establish a mechanistic relation. AFP bias could affect plasticity in several ways: bias might be necessary and sufficient to guide plasticity; bias could guide plasticity but only in the presence of a third signal either from LMAN or perhaps a neuromodulator (Salgado-Commissariat, Rosenfield et al. 2004); or bias might simply make the exploration of motor space more efficient but otherwise be completely unrelated to motor pathway plasticity.

Hebbian plasticity would suffice for consolidation

There is a simple mechanistic explanation of how AFP bias could be both necessary and sufficient to guide plasticity. Hebbian plasticity postulates that the coincident activity of pre- and post-synaptic neurons causes a strengthening of their synaptic connections (Hebb 1949). Imagine that in order to improve imitation, premotor drive from LMAN biases a particular RA neuron to fire, more often than not, at a particular time in the song motif. Since each HVC neuron bursts at a single time within

the motif (Hahnloser, Kozhevnikov et al. 2002), a small subset of HVC neurons will burst at only this same time. Hebbian plasticity will cause any synaptic connection between this subset of HVC neurons and the RA neuron to be strengthened. Over time these HVC cells will come to drive the RA neuron to fire *without input from LMAN*. In other words, the bias from LMAN will have been consolidated into the motor pathway.

One experimental test of this hypothesis involves artificially driving correlated LMAN activity at a particular time in the motif using electrical stimulation. Stimulation will result in a transient perturbation of the song (Kao, 2005). If time-locked drive from LMAN were necessary and sufficient to produce motor pathway plasticity, then the changes observed in the song following stimulation should, over time, begin to appear even without stimulation. This experiment was conducted in one bird and gave a negative result (Fig 2). Stimulation of LMAN time-locked to a particular moment in the song (with 6ms jitter) caused a clear and consistent spectral change in the song. However, after eight days of motif-locked stimulation (on 80% of motif renditions), the pair-wise similarity between syllables produced at the end of the eighth day (without stimulation) and the pre-experimental syllables (84.5 ±7.7 s.d.) was not significantly different than the pair-wise similarity within the pre-experimental group (84.9 ±11.3 s.d.; $p = 0.23$, two-tailed t-test) - indicating that time-locked stimulation caused no change in the syllable.

The failure of this experiment to alter the targeted syllable suggests that 1) AFP bias is not sufficient to drive plasticity, or that 2) the correlated LMAN activity induced by electrical stimulation was not sufficiently realistic to drive plasticity. An example of

the latter would include the possibility that correlated LMAN activity must persist outside of singing, during offline replay (Wilson and McNaughton 1994; Dave and Margoliash 2000), in order to induce motor pathway plasticity. Given these various interpretations, it remains an open question whether AFP bias is sufficient to drive plasticity. Nonetheless, the correlation presented in chapter 4 renders the AFP an exciting new circuit in which to study memory consolidation.

When does motor pathway plasticity occur? Does it occur at night?

Given the body of evidence linking the consolidation of memories to sleep (Dave and Margoliash 2000; Walker, Brakefield et al. 2003; Deregnaucourt, Mitra et al. 2005), another important question is what happens to AFP bias and motor pathway plasticity overnight. One possibility is that the AFP may begin each morning providing zero bias, and that experience-dependent accumulation of AFP bias accounts for all of the learning that occurs during the day (Fig. 3A). This would imply that the consolidation of AFP bias into the motor pathway occurs exclusively at night. The possibility was tested by measuring AFP bias in the morning after a prior day of learning. (n=2 birds, 11 additional experimental days, inactivation on alternate days). Inactivation of LMAN caused a regression of syllable pitch opposite the direction of learning the previous day (Fig. 3C and D), showing that the AFP generates a significant pitch bias in the morning. The AFP bias in the morning (Fig. 3D, slope=-0.70±0.15, $r^2=0.70$, $p<0.001$, slope less than zero) was nearly as large as with evening inactivation (chapter 4). Since increasing AFP bias cannot therefore account for all of the learning observed during the day, plasticity must

be occurring in the motor pathway during the day. In other words, these preliminary data suggest that consolidation in this particular system does not occur exclusively at night.

Relation to the basal ganglia and reinforcement learning

The AFP is homologous to the basal ganglia-thalamocortical loops found in the mammalian brain. As discussed in the first chapter, these loops are important for certain types of learning (Packard and Knowlton 2002). However, the specific functions served by these loops remain far from clear. The model of song learning described and supported in this chapter suggests several novel functions of basal ganglia-thalamocortical circuitry. These functions map closely onto the essential components of reinforcement learning: exploratory behavior and the evaluation of behavior outcomes. This model suggests that basal ganglia-forebrain circuits are directly involved in generating exploration; and that they use the evaluation of past performance to incrementally improve motor programs by generating temporally precise premotor drive.

The basal ganglia are involved in learning tasks that require non-declarative memory (Packard and Knowlton 2002). This category of tasks includes motor learning, habit formation, and perceptual skill acquisition. In all cases, non-declarative learning is expressed in action or performance, rather than words or recollections (Squire and Kandel 2008). A characteristic of this type of learning is that it occurs slowly and only with repetition (Squire 1992; Karni 1996). This fits with the generally accepted belief that non-declarative learning proceeds by trial and error, or reinforcement, learning (Sutton and Barto 1998; Wolpert, Ghahramani et al. 2001).

A dedicated circuit for the generation of variable behavior

When learning by reinforcement, the generation of trials that produce different outcomes is just as important as the assessment of the outcome of individual trials. Without variation in behavior, reinforcement learning can't proceed. Yet the neural substrates of exploration have been largely neglected (Daw, O'Doherty et al. 2006).

The experiments described in this dissertation demonstrate that basal ganglia-thalamocortical loops are involved in the active generation of exploratory behavior (chapter 2 and 3). This idea is a novel one. Variable behavior could be generated by noisy processes within motor circuitry, like stochastic vesicle release (Seung 2003); but dedicating a separate neural circuit to the task offers important advantages: the amount of variability can be easily increased or decreased; variability can be focused on certain aspects of a behavior; variability can be shaped so that motor space is explored efficiently; and an efference copy of the variability can be easily generated and processed by other brain regions. It will be interesting to see if any of these advantages bear out in future research.

The evaluation of feedback and corrective premotor drive

Unlike the topic of exploration, the topic of how behavioral outcomes are evaluated by the brain is the subject of significant research. Numerous studies link the basal ganglia to this process of evaluation. Functional imaging studies show that blood flow in the striatum is modulated by the amount of reward an action produces (Delgado,

Locke et al. 2003; McClure, Berns et al. 2003; Tanaka, Doya et al. 2004). Electrophysiology in the striatum shows that neurons encode for action value (Samejima, Ueda et al. 2005) and are modulated by expected reward (Kawagoe, Takikawa et al. 1998); and the activation patterns of striatal neurons change as a task is learned (Jog, Kubota et al. 1999; Barnes, Kubota et al. 2005). Additionally, the large dopaminergic projection into the striatum encodes a signal thought to be critical for reinforcement learning – reward prediction error (Sutton and Barto 1998; Schultz and Dickinson 2000); and this dopaminergic input is capable of mediating plasticity (Reynolds, Hyland et al. 2001).

While this evidence strongly suggests that the basal ganglia play an important role in evaluating feedback from behavior, these studies fail to address whether or how this evaluation affects future behavior. The experiments described in chapter 4 provide this critical link to behavior. Inactivation of the avian basal ganglia-thalamocortical circuit revealed that this circuit was responsible for improvements in vocal output. This finding strongly suggests that basal ganglia-forebrain circuitry affects complex motor behavior by providing premotor drive which incrementally improves performance.

A recent study in rodents also demonstrated the importance of the basal ganglia in the expression of a recently learned behavior (Atallah, Lopez-Paniagua et al. 2007); however this study involved a task that simply required deciding whether to turn left or right at a choice point. Singing behavior in the songbird is a more complex motor behavior that involves precisely coordinating and controlling the contractions of

numerous muscles. Observing the behavioral effect of basal ganglia-forebrain circuitry during song learning provides unique insight into the ability of these circuits to correct precisely timed and finely calibrated motor gestures.

Implications for models of basal ganglia computation

A remarkably large and diverse set of models have been proposed regarding the computations performed by the basal ganglia with regard to reinforcement learning. Many of these models are termed actor-critic models; they involve assigning one portion of the basal ganglia to selecting actions (the actor) and another to critiquing action outcomes (the critic) (Joel, Niv et al. 2002). These models, however, often lack in detail and supporting evidence. The model of AFP function proposed in this chapter suggests an interesting computational function for the basal ganglia in songbirds.

The output axons of the AFP, from LMAN to RA, convey a signal with drives variable singing (chapters 2 and 3). These same axons bifurcate and send a second axon branch back to the AFP to the avian basal ganglia homologue, Area X (Nixdorf-Bergweiler, Lips et al. 1995; Vates and Nottebohm 1995). Thus this second branch provides an efference copy of the change in song added to the motor pathway by the AFP – an efference copy of song explorations. Thus Area X is perfectly positioned to compute the correlation between this motor exploration and the dopaminergic input it receives from VTA, an input known to carry reward related information in other species (Schultz and Dickinson 2000). By computing this correlation, Area X could reinforce song

explorations that yielded increased reward so that the AFP produced those explorations more often – thus yielding the corrective bias observed in chapter 4.

This simple view of the computation performed by the avian basal ganglia suggests 1) that the actor incrementally improves actions rather than selects them and 2) that the critic correlates reward with variations in actions rather than complete motor programs.

Conclusion

In summary, the experiments presented in this thesis ascribe new and important functions to the anterior forebrain pathway for vocal learning in songbirds. By analogy, basal ganglia-thalamocortical loops may serve similar functions for motor learning in mammals. The data suggest that these loops have two important functions in the context of reinforcement learning: to drive variable behavior and to bias future behavior incrementally toward better performance.

These findings suggest several potentially fruitful questions for future research. Foremost, what computation does the anterior forebrain pathway perform in order to generate corrective premotor bias? And how do the incremental changes produced by the anterior forebrain pathway get consolidated into the motor pathway? The songbird will certainly prove an invaluable system in which to better understand how motor learning works – how the brain uses practice of complex motor skills to determine how 100 billion neurons in the brain should be modified to do better next time.

Methods

Time-Locked Stimulation of LMAN

Subject. A juvenile, male zebra finch (Taeniopygia guttata), 68 days post-hatch (dph) (bird identifier: aa67). All procedures were approved by the Massachusetts Institute of Technology Committee on Animal Care.

Implantation of stimulators. The bird was anesthetized with isoflurane (2% in Oxygen) and placed in a stereotaxic apparatus (MyNeurolab.com). RA was localized in the right hemisphere using its electrophysiological signature. Stimulation of RA was used to antidromically localize LMAN in the right hemisphere. Bipolar stimulating electrodes (each composed of two 50μm Teflon-coated stainless steel wires separated by 300 μm) were implanted at the center of the localized location and at its reflected location on the opposite hemisphere. The craniotomies were covered with a thin layer of Kwik-Kast (WPI, Inc.), and the stimulating electrodes were secured to the skull with dental acrylic.

Experimental Protocol. After recovery from surgery the bird was connected to a custom electrical commutator via a flexible cable. Custom software running on a digital signal processor (RX8, Tucker Davis Technologies) was used to monitor the song in real time, and the ratio of power in band-pass filters was used to detect a single moment within the song motif (6ms RMS jitter). All singing was recorded using custom Matlab software. From 81 dph until 89 dph, 80% of DSP-detected moments in the song resulted in bilateral stimulation (Iso-flex stimulation isolator, AMPI) of LMAN using ten 40μs pulses of 40μA at 2.5ms intervals. The remaining DSP-detected moments were used as catch trials to observe the song without stimulation.

Data Analysis. 25 consecutive catch-trial renditions of the syllable targeted with stimulation were extracted by hand from sound file recorded on 80 dph and 89 dph. The average pair-wise similarity of these syllables within day and across days was computed using Sound Analysis Pro 1.04 software.

Morning inactivation of LMAN

Subjects. 2 young adult (age 102-182 dph) male zebra finches (Taeniopygia guttata) were used. All procedures were approved by the Massachusetts Institute of Technology Committee on Animal Care.

Methods. All methods were exactly as described in Chapter 4, except as below:

Rather than vehicle and TTX being infused on alternate days, only TTX was infused every other day. On days when TTX was infused the pitch threshold for conditional auditory feedback was not changed from its values on the previous day. Inactivation with TTX was conducted after approximately the first 100 renditions of the song motif in the morning, instead of after 4 hours.

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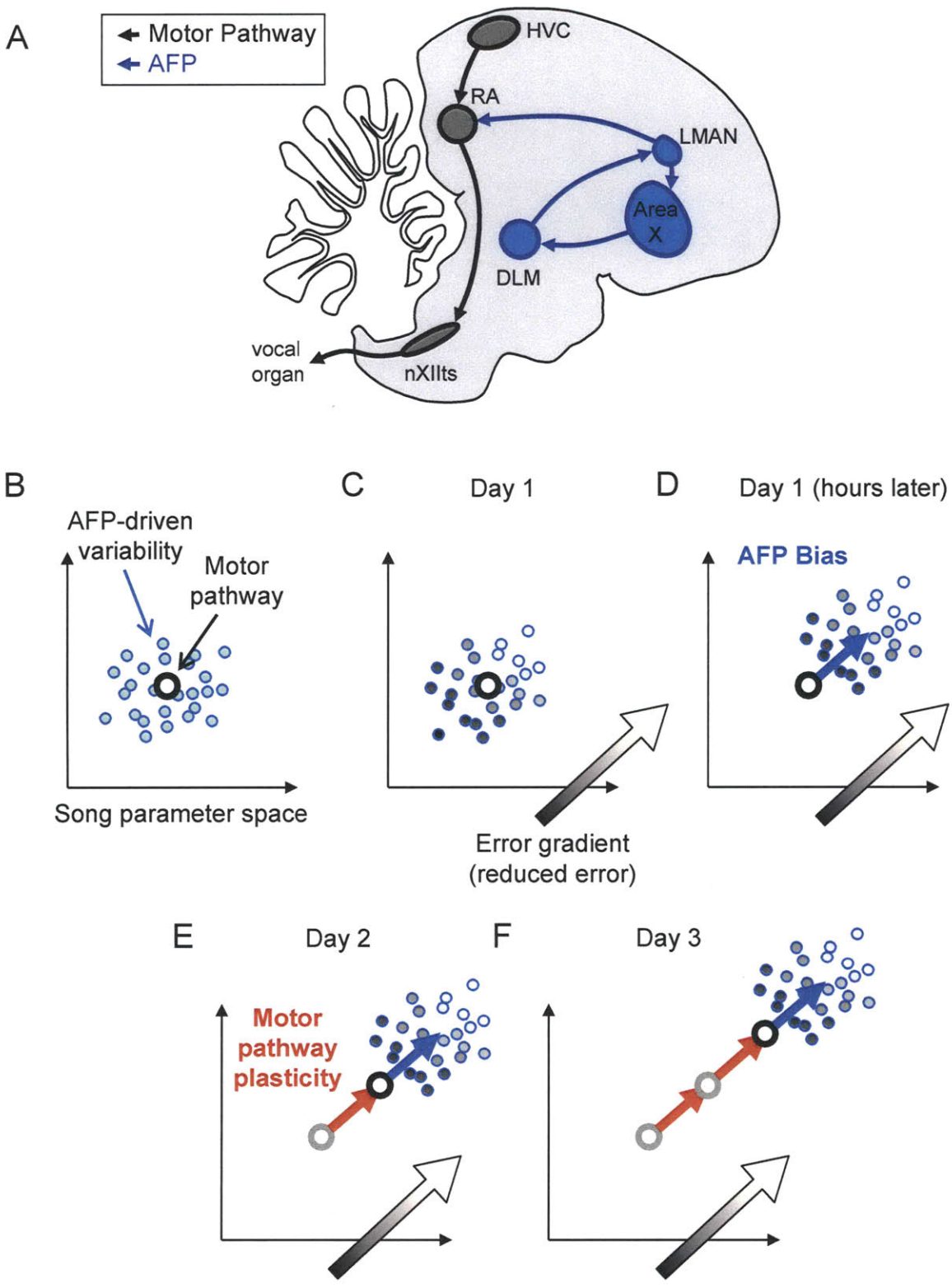
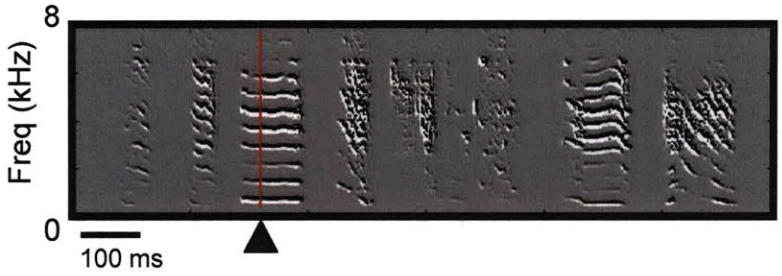

A
Motor Pathway
AFP
HVC
RA
LMAN
Area X
DLM
vocal organ
nXIIts
B
AFP-driven variability
Motor pathway
Song parameter space
C
Day 1
Error gradient (reduced error)
D
Day 1 (hours later)
AFP Bias
E
Day 2
Motor pathway plasticity
F
Day 3

Figure 1. A simple model of how the two major neural pathways in the song system differentially contribute to vocal learning. **A)** Schematic of the two pathways: the motor pathway (black) includes motor cortex analogues HVC and RA, while the anterior forebrain pathway (AFP, blue), a basal ganglia-thalamocortical circuit, consists of Area X, the dorsolateral anterior thalamic nucleus (DLM), and LMAN, which, in turn, projects to RA. RA projects to the motor nucleus (nXIIts, nucleus of the twelfth nerve) which controls vocal output. **B)** In this model of song learning, the space of all song is represented as a simple Cartesian coordinate system. The song encoded by motor pathway is represented by a single point (black circle) because it is relatively stereotyped. The AFP introduces variability into the song, therefore the songs rendered when the AFP is intact are scattered about the version encoded by the motor pathway (small blue dots). **C)** Variations in song produce auditory feedback that varies in its similarity to the template song (better imitation represented by lighter shading of small dots). A gradient of imitation quality is discovered. **D)** On the timescale of hours the presence of this gradient causes the variability driven by the AFP to be become biased in such a way that the average quality of imitation is improved (blue arrow). **E)** On the timescale of about a day, the AFP bias causes plasticity to occur in the motor pathway of a magnitude equal to the size of the bias. **F)** The process continues over multiple days until a good imitation of the template song is produced.

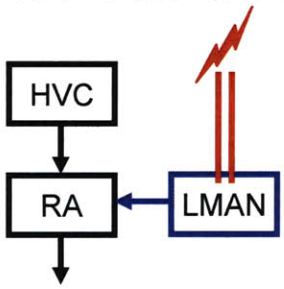
A

1. Bird sings



2. Computer detects targeted moment in the song.

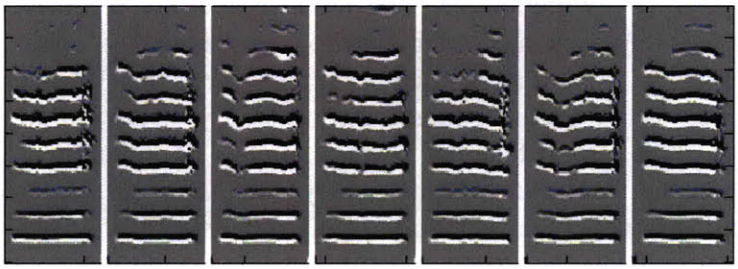
3. Detection triggers bilateral electrical simulation of LMAN.



vocalization

B

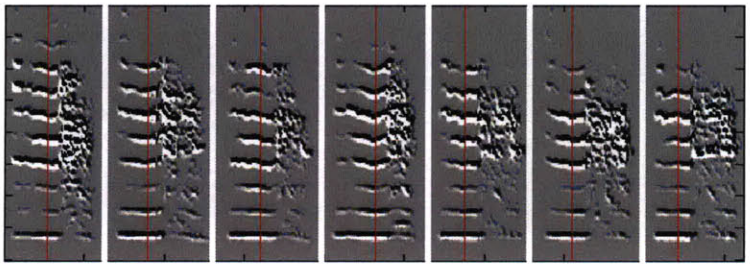
Prior to stimulation (80 dph)



84.9 ±11.3
Mean pairwise similarity within set.

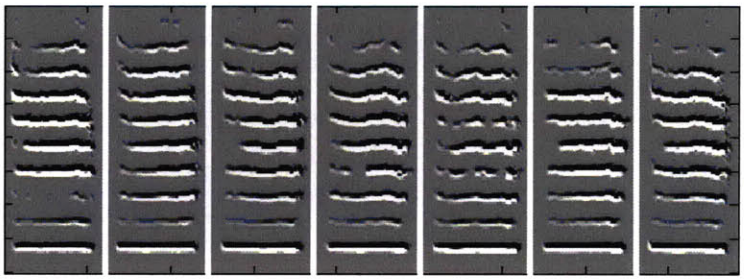
C

Eight days of stimulation (80% of renditions; 81-88 dph)



D

Catch trials on 9th day of stimulation (89 dph)



84.5 ±7.7
Mean pairwise similarity with prestimulation set

Figure 2. Preliminary data suggesting that syllable-locked LMAN stimulation does not induce song changes. **A)** A digital signal processor is used to detect when the bird reaches a particular moment in its motif (with 6ms RMS jitter). Eighty percent of detections trigger bilateral stimulation (ten 40μs pulses with 40μA at 2.5ms intervals). Twenty percent of detections do not invoke stimulation (catch trials). **B)** Seven renditions of the targeted syllable targeted before the beginning of the syllable-locked LMAN stimulation protocol. **C)** Seven renditions of the syllable showing the effect that LMAN stimulation had on vocal output. **D)** Seven renditions of the syllable during catch trials following eight days of syllable-locked LMAN stimulation. The pair-wise similarity between post-protocol syllables and pre-protocol syllables was not significantly different from the pair-wise similarity within the pre-protocol syllables ($p = .23$, two-tailed t-test). This suggests that the song encoded by the motor pathway was unchanged by time-locked LMAN stimulation.

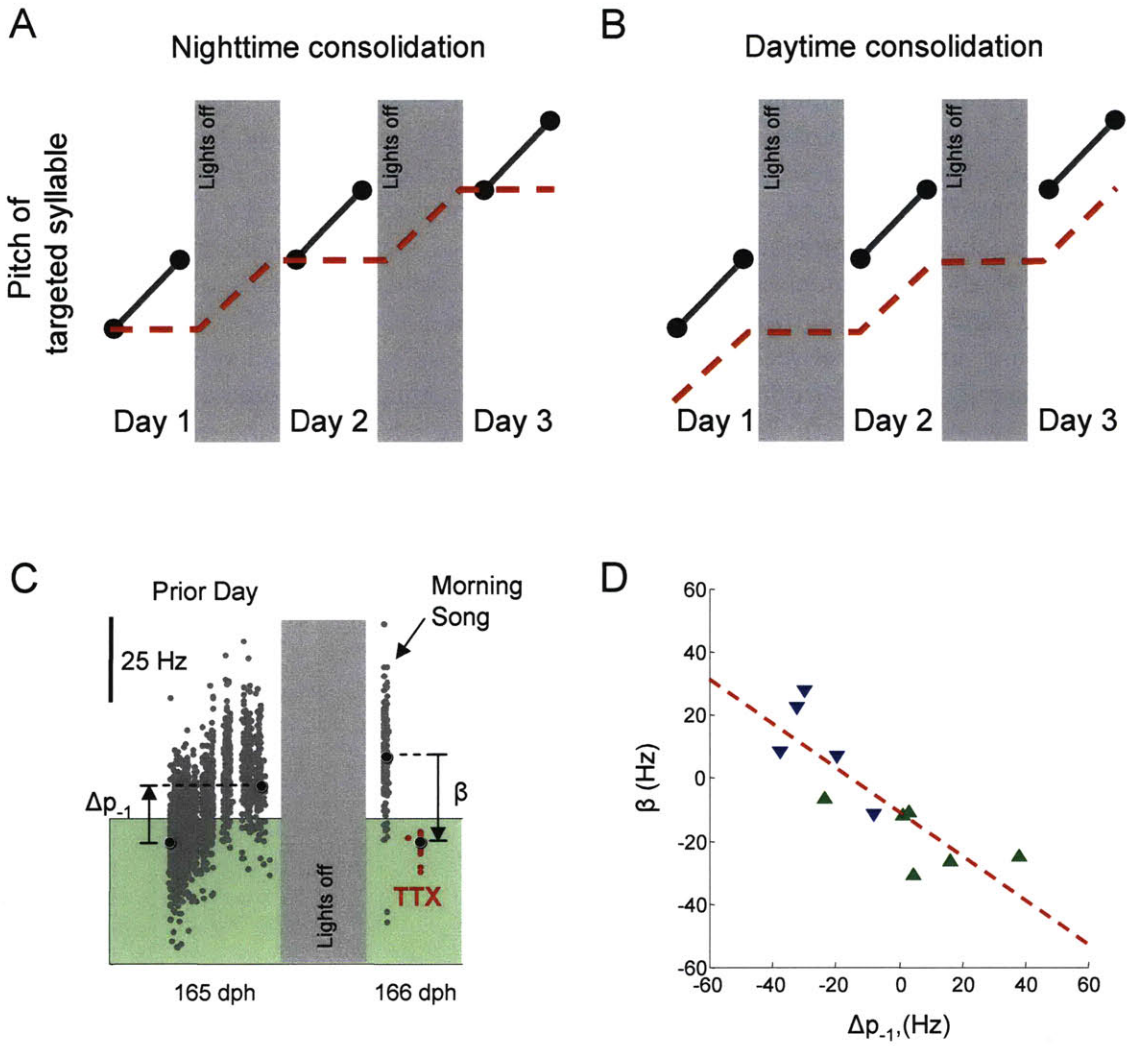

A
Nighttime consolidation
Pitch of targeted syllable
Lights off
Lights off
Day 1
Day 2
Day 3
B
Daytime consolidation
Lights off
Lights off
Day 1
Day 2
Day 3
C
Prior Day
Morning Song
25 Hz
Δp-1
β
TTX
Lights off
165 dph
166 dph
D
β (Hz)
Δp-1,(Hz)
60
40
20
0
-20
-40
-60
-60
-40
-20
0
20
40
60

Figure 3. Preliminary data suggesting that AFP bias persists after nighttime sleep. **A)** Schematic of the nighttime consolidation hypothesis. Black lines indicate the pitch of targeted syllable produced by the intact song system. Dashed red lines indicate the pitch encoded by the motor pathway (when LMAN is inactiviated). AFP bias changes during the day, and these changes are consolidated into the motor pathway at night. **B)** Schematic of the daytime consolidation hypothesis. Note that inactivation in the evening does not distinguish the nighttime and daytime hypotheses, but inactivation in the morning does. **C)** Preliminary data from morning inactivation of LMAN. During a day of conditional auditory feedback (described in chapter 4) the bird raises the pitch of the targeted syllable (average pitch of individual syllables, gray dots; Δp-1 is the difference between the mean pitch of first and last 50 syllables of the day, black dots). After a 12h dark cycle, LMAN is inactivated in the morning (after ~100 song renditions) with tetrodotoxin (TTX). Inactivation in the morning produces a change of syllable pitch opposite the direction of on-going learning (average pitch of individual syllables during inactivation, red dots; β is the difference between the mean pitch of the first 50 syllables after inactivation and the last 50 syllables before inactivation), which is not predicted by the nighttime consolidation hypothesis. **D)** Scatter plot of the pitch regression following morning inactivation (β) versus the pitch change on the previous day (Δp-1). The negative correlation indicates that AFP bias persists following nighttime sleep (linear regression, red line; slope=-0.70±0.15, r2=0.70, $p<0.001$, slope less than zero).

Appendix A

A novel method for the manipulation of auditory feedback in songbirds

Abstract

Discovering how the brain uses practice of a behavior to improve future performance is fundamental to understanding motor learning. The outcome of an action, for instance whether a tennis serve was successful, must be used to determine which of a 100 billion neurons in the brain should be modified. Vocal learning in the songbird is well suited for the study of this question. The bird learns its song by practicing and using the resulting auditory feedback. Manipulating this auditory feedback has proved valuable in understanding how it is processed by the brain. This appendix describes a novel technique for manipulating auditory feedback in songbirds without disrupting the ability to record the bird's vocalizations. This method exploits a previously unappreciated aspect of the bird's anatomy: the interior surface of both eardrums is continuous with a cranial air-sac located between the two layers of the skull. By implanting a small hearing-aid speaker into this air-sac, sounds can be played to the bird that are not detected by an external microphone. This method enable experiments in which feedback is a online function of singing. It also eliminates the need for catch trials and difficult post-hoc removal of feedback from recordings.

1. Introduction

A wide range of techniques have been used to manipulate auditory feedback in songbirds. These include: deafening (Konishi 1965; Brainard and Doupe 2000); damaging the tracheal syringeal nerve to distort vocalizations (Williams and McKibben 1992; Williams and Mehta 1999); reversible deafening using antibiotics (Woolley and Rubel 2002); partial-muting (Cooper and Goller 2004); temporary muscle paralysis (Pytte and Suthers 2000); and manipulation of environmental gases (Nowicki 1987).

In addition, speakers have been used to manipulate auditory feedback while birds sing. The speakers are controlled by a computer which enables specific spectral and temporal components of the song to be manipulated (Leonardo and Konishi 1999). This approach has proved useful for probing how and when both neural activity (Leonardo 2004; Kozhevnikov and Fee 2007; Sakata and Brainard 2008) and song (Leonardo and Konishi 1999; Sakata and Brainard 2006) change in response to feedback. However, this approach has two major limitations. First, this method of manipulation interferes with the recording of the bird's vocalizations, which prevents continuous monitoring of singing and results in an imperfect dataset. Second, the manipulation is affected by the bird's position and orientation relative to the speaker – creating an artificial dependence.

We address these limitations by developing a novel method for the manipulation of auditory feedback in songbirds. The method capitalizes on the unique anatomy of the avian skull to create what are in essence zebra finch "headphones." This technique

allows auditory feedback to be manipulated independent of the bird's position and without impact on the recording of the bird's vocalizations.

2. Methods

2.1. *Speaker implant*

In order to optimize their weight, birds have evolved skulls that comprise two thin layers of bone separated by an airspace and held together by thin trabecula (Feduccia 1999). In the zebra finch (*Taeneopygia guttata*, a small songbird weighing 12-15g) this airspace is enlarged in the posterior portion of the skull on both sides of the cerebellum (Fig. 1A,B). A computed tomographic scan of the zebra finch head, as well as anatomical exploration during deafening experiments, determined that this enlarged airspace is continuous with the internal surface of both eardrums (Fig. 1C). Therefore, modulation of pressure within this airspace will result in vibration of the eardrum, the same way that modulation of external air pressure vibrates the eardrum.

2.2. *Speaker implant construction*

To exploit this anatomical fact, the researcher designed an implantable speaker capable of manipulating the air pressure in the cranial airspace (Fig. 2). The implant consisted primarily of a small hearing-aid speaker (WBHC-23910, Knowles Electronics, 0.21 g, 5.17 x 3.55 x 3.00 mm) and a small electrical connector (Nano Series, Omnetics Corp.) through which to drive the speaker. Biocompatible tubing (MicroRenathane Implantation Tubing, Braintree Scientific, Inc., 0.025" O.D.) was connected to the speaker and used to pipe sound into the cranial airspace. This tubing was inserted into the speaker opening

and secured with a concentric fitting tube and cyanoacrylate. A small microphone (EM-23046-CX, Knowles Electronics, 0.08g) was also attached to the implant. This microphone provided position invariant recordings of the bird's vocalization that were minimally susceptible to resonances in the cage.

To prevent condensation from accumulating inside the speaker and dampening its output, the speaker was constantly kept warmed to slightly above body temperature using ohmic heating. Three 100 Ohm chip resistors were glued along the body of the speaker and attached in series. They were electrically insulated from the speaker using a thin layer of silicon elastomer (Kwik-Cast, WPI Inc). A constant voltage was applied across these resistors following implantation in order to keep the speaker at approximately 43 degrees Celsius.

2.3. *Implantation of the speaker tube*

Subjects were older juvenile and young adult male zebra finches (Taeneopygia guttata, 70-150 days post-hatch), 12-15g in weight. Birds were anesthetized with 1-2% isoflurane and a craniotomy was made in the outer layer of the skull with a radius of 0.8 mm. The location of the craniotomy was approximately 2.5 mm lateral and 2.0 mm posterior of lambda, at 54 degrees, anterior portion of skull relative to horizontal. The location was optimized by backlighting the skull in order to estimate where the spacing between the inner and outer layers was largest.

After opening the outer layer of the skull, the trabecula were carefully removed using sterile forceps. It was important that no blood or fluid accumulate in the airspace during or after implantation, as this could clog the speaker tube. Any blood or fluid that did result from removal of trabecula was immediately absorbed using a sterile kimwipe. The trabecula were removed down to a depth approximately even with the vestibular canals, which are visible within the cranial airspace. A space was cleared to accommodate the speaker tube without any bone or trabecula contacting it. The desired angle and depth of the implant were carefully estimated and the speaker tube was lowered to approximately 0.75mm from the dorsal surface of the vestibular canal. The ring of air between the outer layer of skull and the speaker tube was sealed with silicon elastomer (Kwik-Cast, WPI Inc.), and the implant affixed to the skull using dental acrylic (Flow-It ALC, Pentron Clinical Technologies). The weight of the implant, including dental acrylic, was between 0.7 and 0.9 g.

2.4. *Calibration of the speaker implant*

The precise manipulation of auditory feedback requires that the sound level of the speaker be calibrated. For each implant, the transfer function between the implanted speaker and the sound pressure level in the cranial airspace was measured. A second craniotomy was made in the outer layer of the skull at the laterally reflected location of the implant. The craniotomy was made just large enough to accommodate the tip of a calibrated probe microphone (ER-7C, Etymotic Research). A broadband white noise signal (40 kHz sampling rate) was used to drive the speaker. Both the noise and the response of the microphone were recorded and subsequently used to compute the

transfer function (Fig. 3A). This transfer function allows the loudness of feedback to be precisely set and the feedback to be whitened or filtered to account for the non-uniform spectrum of the transfer function. The second craniotomy was sealed with silicon elastomer and covered with dental acrylic.

2.5. *Confirming speaker effectiveness*

After calibrating the speaker, a second measurement of speaker effectiveness was performed by placing the tip of the probe microphone into the left ear of the bird. Again, broadband white noise was used to drive the speaker, and the response of the in-ear microphone was recorded (Fig. 3B).

To ensure the continued effectiveness of the speaker, the bird was re-anesthetized at the end of the experiment, and the in-ear transfer function re-computed. While this transfer depends slightly on the exact position of the probe microphone within the ear, it proved sufficient to verify that the speaker continued to function.

2.6. *Controlling the speaker and using the device*

After surgery, the bird was placed in a sound attenuating chamber and allowed several days to recover. After recovery, the bird was connected to an electrical commutator (SL-88, Dragonfly Inc.) via a flexible cable. Several signals were passed via the commutator to and from the implant: the signal to drive the speaker, a constant voltage (6.5 V) to warm the speaker, power and ground for the microphone, and the microphone output signal.

To test the device, custom MATLAB (Mathworks) software was used to simultaneously record the microphone signal and control the implanted speaker. All signals played out of the speaker were whitened using the inverse of the in-skull calibration transfer function between 0.4 and 8 kHz.

3. Results

The implanted speaker device was used to manipulate the sounds perceived by a zebra finch. The birds tolerated the implant well. The implant weighed only 0.7-0.9 g (n = 10 birds) which is significantly lighter than the motorized microdrive previously used with zebra finches (Fee and Leonardo 2001). In addition, the surgery was shorter and less invasive than microdrive implantation, involving only removing the outer leaflet of skull and trabecula. After the birds acclimated to the tether and commutator system, the birds sang both directed and undirected song.

To determine if the birds perceived the sounds played into the skull, we tested whether male birds responded to female distance calls played through the speaker. Distance calls generally invoke an immediate distance call reply (Zann 1996). Indeed the implanted males responded promptly when female calls were played into the speaker (Fig. 5). The perception of the feedback was also confirmed by the successful use of the device to induce learning (Chapter 4).

This test also confirmed that the microphone was not sensitive to sounds produced by the speaker. Female calls were played as loud as 95 dB and no response was recorded by the cage microphone.

Lastly, we confirmed that the device continued to function for several weeks after implantation. The amplitude of the in-ear calibration response 26 days after implant was reduced compared to the amplitude on the day of surgery. This was true for the majority of birds. However, in 3 of 10 birds, in-ear calibrations showed significantly reduced amplitude post surgery. It was confirmed that in these three birds the speaker tube had become clogged.

4. Discussion

The implanted speaker device allows auditory feedback to be manipulated without contaminating real-time monitoring of zebra finch vocalizations. Therefore feedback can be a function of ongoing singing in ways not previously possible. Complex relationships been feedback and ongoing singing have proved useful in recent experiments (Andalman and Fee 2009; Sober and Brainard 2009) and will likely continue to prove useful in the future.

Other approaches have attempted to prevent auditory feedback manipulation from contaminating song recordings. One such technique is active sound cancellation (Leonardo 2004). This technique removes the contaminating sound from the previously recorded file using post-hoc computations. Sound cancellation, however, is imperfect and

it has to be performed off-line, precluding experiments that require real-time monitoring of song.

Another recent technique used to prevent contamination of song recordings involved building finch headphones that fit externally over the ears of the bird (Sober and Brainard 2009). Here vocalization can be monitored in real-time while manipulating feedback. And, as with the implanted speaker, auditory feedback is independent of the bird's position. The primary advantage of finch headphones compared to the implanted speaker device are that they are not subject to potential clogging.

However, since zebra finch headphones are larger, heavier, and more difficult to construct than the implanted speaker device, the implant speaker device will prove more practical for many applications.

Acknowledgements

We thank Ricardo Gonzalez Rubio for producing the CT-scan of the zebra finch head.

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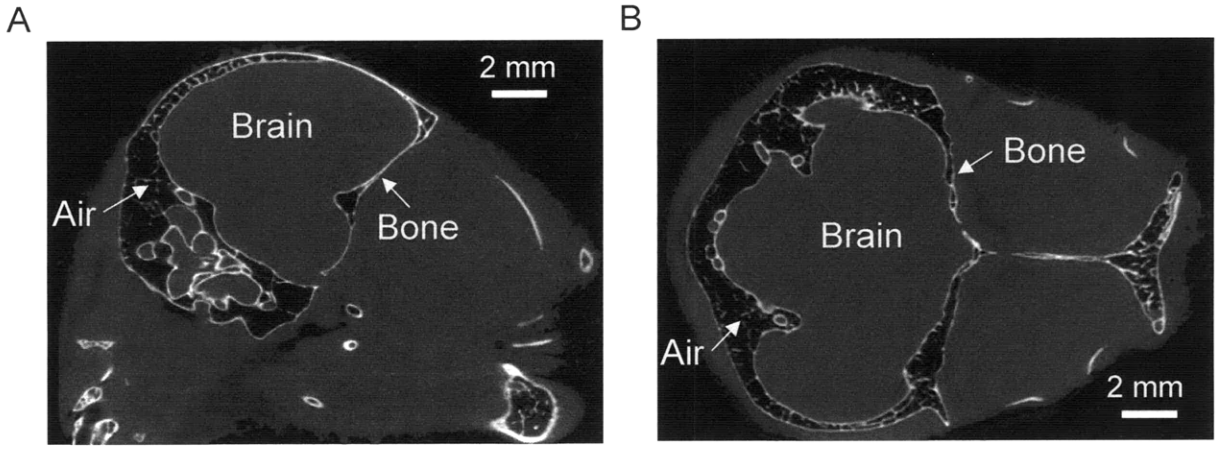

A
2 mm
Brain
Air
Bone
B
Bone
Brain
Air
2 mm

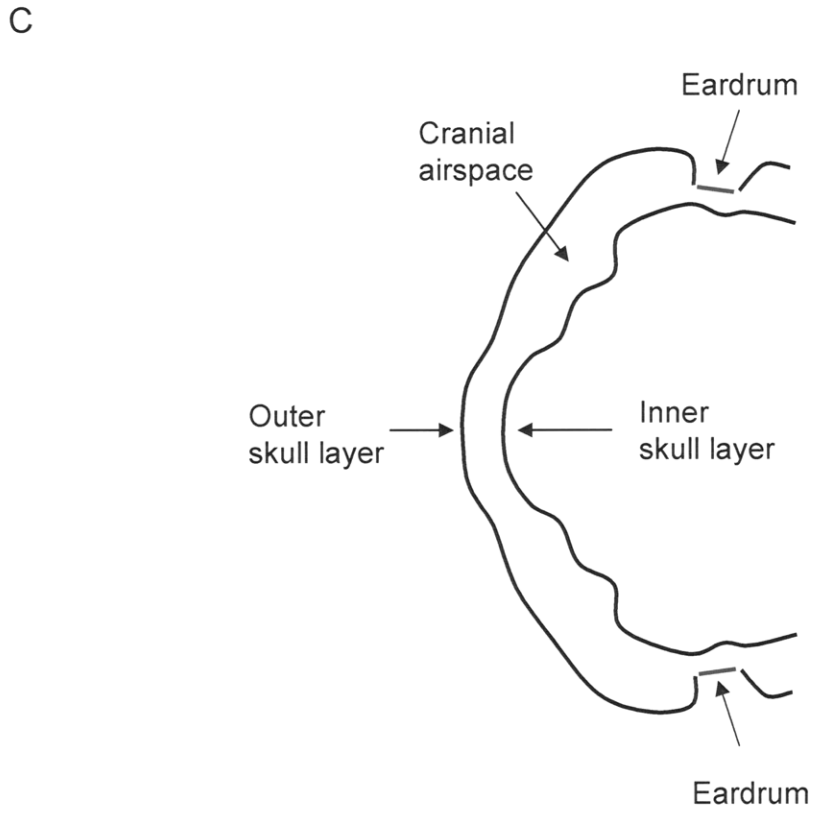

C
Eardrum
Cranial airspace
Outer skull layer
Inner skull layer
Eardrum

Figure 1. The airspace between the inner and outer layer of skull is continuous with the interior surface of the eardrums. **A)** Parasagittal section of a computed tomography scan of the zebra finch head showing the enlarged airspace lateral of the cerebellum. **B)** Horizontal section showing the same airspace. **C)** Schematic illustrating the continuity of the intra-skull airspace with interior surface of the eardrums.

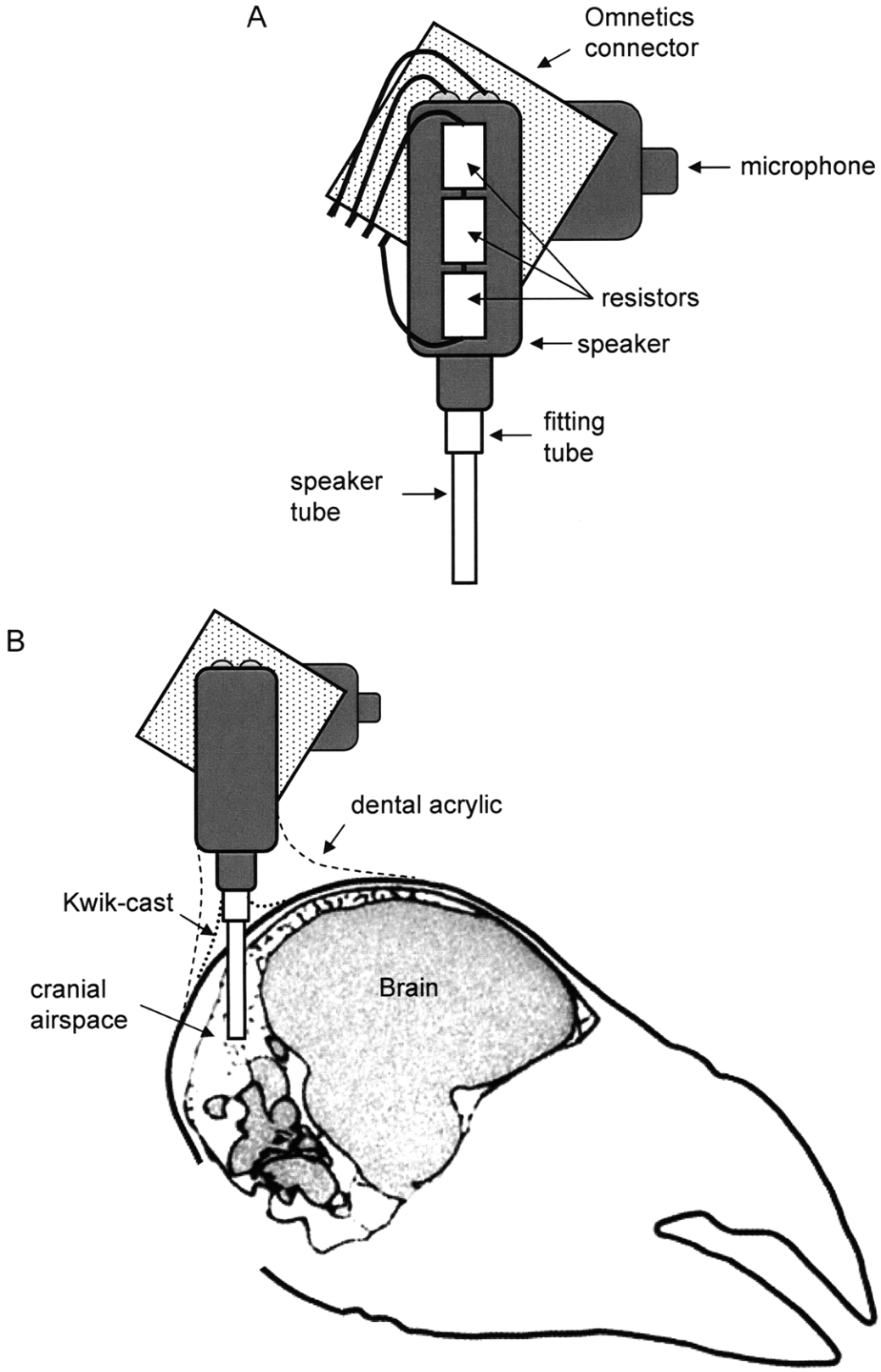
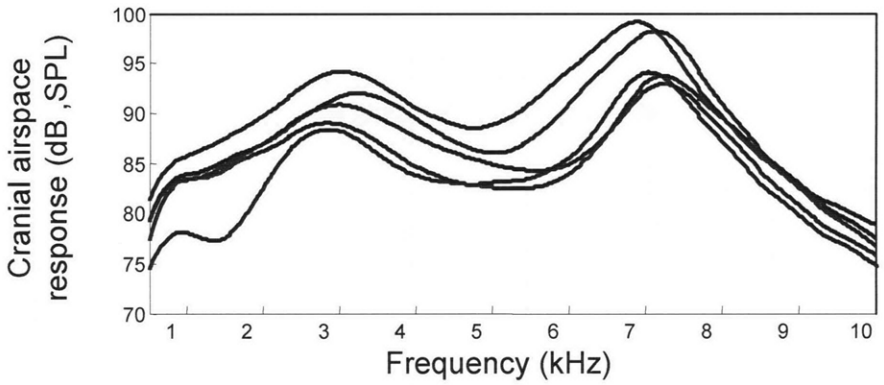

A
Omnetics
connector
microphone
resistors
speaker
fitting
tube
speaker
tube
B
dental acrylic
Kwik-cast
cranial
airspace
Brain

Figure 2. The implantable speaker device. **A)** Diagram of the construction of the implantable speaker device. **B)** Illustration of how the speaker device is implanted into the cranial air space.

A


Cranial airspace response (dB ,SPL)
100
95
90
85
80
75
70
1
2
3
4
5
6
7
8
9
10
Frequency (kHz)

B

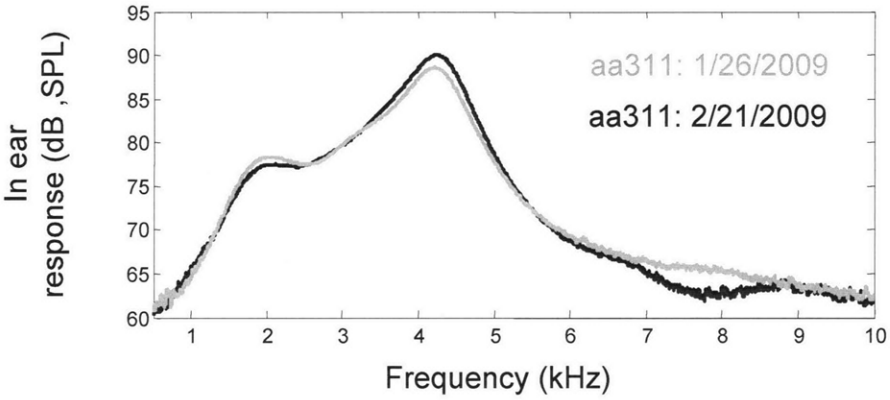

In ear response (dB ,SPL)
95
90
85
80
75
70
65
60
1
2
3
4
5
6
7
8
9
10
Frequency (kHz)
aa311: 1/26/2009
aa311: 2/21/2009

Figure 3. Calibration of the implanted speaker device. **A)** The response of the calibration microphone when placed in cranial airspace of 5 different birds. The implanted speaker was driven by broadband white noise (0.707 mA RMS). **B)** The in-ear response of the calibration microphone immediately following implant (gray) and again 26 days later (black).

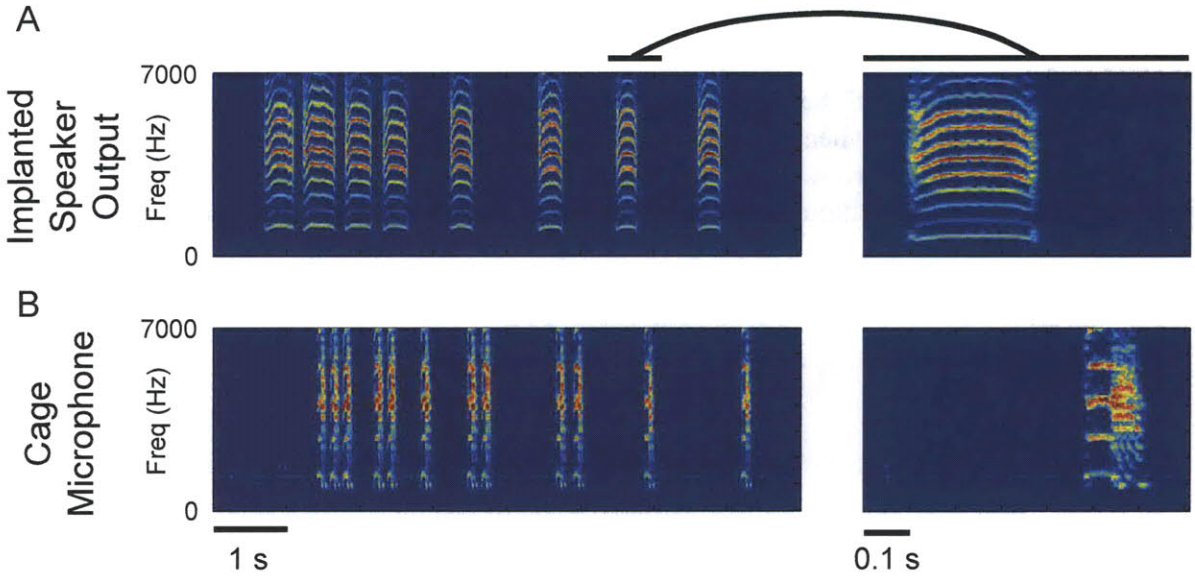

A
Implanted Speaker Output
7000
Freq (Hz)
0
B
Cage Microphone
7000
Freq (Hz)
0
1 s
0.1 s

Figure 4. The implanted speaker device can manipulate auditory feedback without interfering with recording of the bird's vocalizations. **A)** The spectrogram of a sound file played by the implantable speaker. The sound file produces a series of female zebra finch calls at 91 dB. **B)** Spectrogram of the simultaneous recorded cage microphone signal. The female calls are not detected by the microphone, but the calls do provoke a response by the implanted male bird. The male bird's response calls are recorded uncontaminated by the sounds produced by the cranial speaker.