Doctoral Thesis

Study of interhemispheric coordination for birdsong production

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Publication Date:
2010

Permanent Link:
https://doi.org/10.3929/ethz-a-006139049

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STUDY OF INTERHEMISPHERIC COORDINATION
FOR BIRDSONG PRODUCTION

A dissertation submitted to
ETH ZURICH
for the degree of
Doctor of Sciences

presented by
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2010
Acknowledgement

First, I want to express my thanks to all my friends who have lent their support to help me complete this work. My thesis supervisor, Prof. Richard Hahnloser, is a great mentor. His wonderful kindness, great sense of humor, smart working style, strong scientific insights, and English writing ability were significant elements which guided my entire PhD journey at the Institute of Neuroinformatics (INI). I want to thank: Prof. Rodney Douglas, for accepting the position of thesis examiner, and Prof. Walter Senn at University of Bern for his kindness as my co-supervisor as well as for his “Lucky Wish You Well” memo which brought me comfort throughout many intensive PhD research episodes. Former lab technician, Aymeric Nager provided a “Swiss-Quality” construction method that positively influenced me while I worked to understand hardware and lab instrumentation, both of which I discovered to be essential to becoming an experimental neuroscientist. Aymeric’s cheeky sense of humor and patient assistance supported my life at INI. In addition to the professors and staff at INI, I would also like to thanks my doctoral student colleagues. Georg Keller, the very first PhD student from the songbird group, is like a brother to me. While in the lab, his insightful advice taught me to be efficient. Often times, I joked with him regarding this additional stress he placed on me. My major source of happiness came from songbird group member Joshua Herbst, who, in addition to Richard, has been the most important person who helped me be able to publish my research results in a high-impact journal within a reasonably short time. I truly appreciate his good friendship and great help. My “package friends,” Moritz Kirschmann and Daniele Oberti, together with Florian Blaettler, Andreas Kotowicz, Michael Graber, and Janie Ondracek provided delightful companionship. These personality combinations made my time in Switzerland and with INI swift.

I appreciated the secure and comfortable working environment that is guided by Prof. Rodney Douglas and Prof. Kevan Martin’s great leadership. This institute, with its great academic atmosphere, is the best institute I have ever belonged to. Last but not least, I would like to specially thank Kevan for his great sense of humor and kindness shown to me. Every time I encountered him at INI, his humorous regards and greetings always brought me comfort, and probably more than he knew, many times it healed my frustration from some tough research bottlenecks.

I want to dedicate this thesis together with my PhD degree achievement to my parents, especially to my beloved father who passed away in 2007. His love and support secured my growth and strong passion for science. He lives in my heart forever!
I dedicate this thesis to the memory of my father, Lu-Ben Wang.
Abstract

Complex learned motor sequence generation, such as birdsong production, relies on precise coordination of activity in both brain hemispheres; however, little is known about the neural mechanisms of this coordination. By using systematic electrical and visual stimulation to briefly perturb the activity of neurons in the motor pathway during song production, I investigated the neuronal dynamics responsible for generating different acoustic elements of birdsong. These experiments allowed me to dissect complex motor behavior and verify the hypothetical existence of behavioral units for procedural sequences of motor behavior.

On one hand, I discovered mostly disjoint time intervals during which electrical stimulation of either the right or left hemisphere was effective in distorting vocalizations. Such interhemispheric switching of stimulation effectiveness was even observed during repetitive calls uttered immediately after song. This evidence supports a novel form of ping-pong–like motor dynamics for song production. Furthermore, the interhemispheric switching patterns obtained from two sibling birds, both of which learned songs that were temporally and spectrally similar, were intrinsically uncorrelated. This implies that an idiosyncratic neural representation for learned song exists across both cerebral hemispheres.

On the other hand, using non-invasive visual perturbation, I found that song disruptions also occurred in the midst of syllables and that furthermore, certain parts of the song motifs were resistant to visual perturbation. By analyzing syllable transitions for several birds receiving visual or electrical stimulation in HVC (a premotor nucleus for song production) and LMAN (a basal ganglia nucleus for song learning), I found most stimulation-induced syllable transitions were also observed during catch trials, although much more rarely. Therefore, stimulation seems to selectively increase the likelihood of some rare song sequence transitions within the song repertoire. By analyzing the size distribution of an effective set of contiguous stimulation times, I found evidence for power-law behavior and for self-organized chain networks of HVC projection neurons.

Taken together, these findings indicate that the motor program for song production contains discrete sub-syllabic elements that reside bilaterally in the brain and are coordinated via rapid interhemispheric switching dynamics. The formation of the neural networks representing these elements might result from the idiosyncratic song learning process specific to each bird.
Chapter 1: Introduction
- Birds as an ideal animal model for neural process of speech and music occurred in both brain hemispheres.
- Sequential neural processing of language in the human brain.

Chapter 2: Brief Review
- Neural basis and terminology of birdsong.
- Brain lateralization and interhemispheric switching.

Chapter 3: Rapid Interhemispheric Switching Dynamics during Song Production in a Zebra Finch
- Interhemispheric complementary stimulation effectiveness only occurs in singing state, implying a switching dynamics.
- Left and right hemispheres are dominant in processing amplitude-modulation and frequency-modulation of song, respectively.

Chapter 4: (I) HVC Stimulation Evokes Activation in the Contralateral Forebrain Song Nuclei (II) The Thalamus may Gate Interhemispheric Coordination
- Auditory responses in premotor areas HVC and RA can be gated by the thalamic nucleus Uva.
- Identify candidate brain areas and pathways for interhemispheric coordination for birdsong.

Chapter 5: Interhemispheric Switching for Birdsong Production is Uncorrelated with Lateralized Auditory Feedback

Chapter 6: Idiosyncratic Interhemispheric Switching Dynamics for Birdsong Production
- There is no systematic mapping between the interhemispheric switching dynamics and the vocal output of the bird.

Chapter 7: Determination of Unitary Elements of Birdsong
- Unitary song elements may be encoded in self-organized free-scale chain networks of projection neurons in HVC.
- Birdsong represented in the motor program may contain a repertoire of neural networks encoding different sequence transitions. The formation of these networks might be shaped from the idiosyncratic self-organizing song learning process for individual birds.
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Welcome Tomorrow
“As long as our brain is a mystery, the universe, the reflection of the structure of the brain will also be a mystery.”

Santiago Ramón y Cajal (1852-1934)
Chapter 1: Introduction

Serial organization is fundamental to human behavior. Most of our day-to-day activities involve precisely ordered sequences of action. For example, uttering a word requires that syllables are put in the correct order. Forming sentences requires sequencing these words appropriately. Driving a car, serving a tennis ball, and playing the piano are also contingent on learned motor sequences. In 1951, Karl Lashley, a neurophysiologist at Harvard University, highlighted the ubiquity of sequentiality, or serial order in animal motor behavior: “... the coordination of leg movements in insects, the song of birds, the control of trotting and pacing in a gaited horse, the rat running the maze, the architect designing a house, the carpenter sawing a board, present a problem of sequences of action ...” (Lashley 1951).

How motor sequences are represented in the brain has been a long-standing research problem in cognitive science and is currently a major topic in neuroscience. Human speech and music are two of the most remarkable sequential behaviors, and indeed, scientific interest has been increasing in comparative studies between music and human language. It has been shown that brain mechanisms underlying complex sound processing can process syntactic relations in both music and language [see (Patel 2003) as a review]. In Charles Darwin’s book “The Descent of Man, and Selection in Relation to Sex,” published in 1871, he stated, “Primeval man, or rather some early progenitor of man, probably first used his voice in producing true musical cadences, that is in singing.” Therefore the birdsong system, which evolved at least 100 million years earlier than the modern human brain, is a promising candidate for providing us a key framework for understanding the origin of the neural mechanism for complex sequence processing, such as language.

In the animal kingdom, songbirds are one of the few species which learn to sing and have striking parallels to speech and music (Doupe and Kuhl 1999; Goldstein et al. 2003; Rothenberg 2005). Similar to human speech development, birds must hear the sounds of their adult tutors during a sensitive period and must hear their own voice while learning to vocalize a best copy of the tutor song (Immelman 1969). A study of social interaction for shaping babbling first shed light on the general process underlying the

“If I were not a physicist, I would probably be a musician. ... I get the most joy in life out of music.”
Albert Einstein (1879-1955)
development of speech and song (Goldstein et al. 2003). According to the basic definition of music as an organized sequence of sounds, birdsong also shares common structures with music (Rothenberg 2005).

The zebra finch (*Taeniopygia guttata*), a small Australian songbird (Figure 1.1), has been a useful species for determining the neural basis of motor sequence generation and for learning about birdsong. Only male zebra finches sing, and they do this mainly to attract females and repel rivals. Zebra finch songs are not genetically innate but rather are learned from nearby adults, usually from the bird’s father (its tutor). Juvenile birds begin the song learning process with the memorization or sensory phase [~20 days post-hatch (dph)]. They memorize the tutor song as a template and produce noisy vocalizations, which are very similar to human infant babbling (Aronov et al. 2008). Through the reproduction or sensorimotor phase (30~90 dph), they practice their raw vocalization in order to closely imitate the memorized song template. The learning process ends when the juveniles reach adulthood (>90 dph) and with the production of a final version of the accurate copy of the song template.

Figure 1.1. A zebra finch couple and the spectrogram of the male song.

Only the male zebra finch (the left-side colorful bird with zebra stripes on his breast) can sing to a female bird (the mostly grey-colored bird on the right) for courtship. The spectrogram and raw acoustic wave of his song are shown on the right panel.

It may be an exaggeration to say that the lessons learned from a bird brain can directly generalize to a human brain. On the neurophysiological level, speech has been localized to the left-side of the human brain (Broca 1861). However, in the songbird, lesion studies have shown that normal adult birds require both brain hemispheres to produce songs (Ashmore et al. 2007). Linguistically, birdsong is considered to be too simple to be characterized as a form of language and should therefore only be regarded as...
music (Rothenberg 2005). However, it has been suggested that music has an ambivalent relationship with animal songs, and many parallels also exist between music and language at the structural level (Wallin et al. 2001). One could reason that evolution has generated a class of neural information processing systems (such as those used to process animal songs), and even though the details of each species are unique, the general principles that govern their diversified functions (such as the language function) can be quite similar.

From a historical viewpoint, specific behaviors were often thought to be entirely lateralized to a single hemisphere, such as in the case of left-lateralization in language processing (Broca 1861; Geschwind 1970). However, new studies have shown that each hemisphere is not necessary dedicated to a single behavior but is instead specialized for specific features of that behavior (Serrien et al. 2006). In the context of language, the left hemisphere is more specialized for lexical and syntactical processing of language, whereas the right hemisphere is more sensitive to the emotional aspects of speech (Friederici and Alter 2004; Giraud et al. 2007). There is supporting evidence that properties of the auditory cortex are analogous to these specializations. For example, the left auditory cortex is dominant in processing fast temporal changes of sounds that are necessary for the phonological level, while the right auditory cortex is dominant in processing spectral patterns in sounds that are associated with the rhythm, stress and intonation of speech (Giraud et al. 2007; Okamoto et al. 2009).

Music has also been shown to demand bilateral processing. Supporting this, musically trained individuals possess less lateralized function and enhanced interhemispheric interaction (Lin et al. 2002). The anterior corpus callosum is also larger in musicians, possibly reflecting underlying strengthened interhemispheric pathways (Schlaug et al. 1995; Schlaug et al. 2009). Functional neuroimaging studies of sound perception has shown that both brain hemispheres in human infants are involved in the processing of speech, and human adults use both hemispheres to process music (Dehaene-Lambertz et al. 2002; Koelsch et al. 2002; Tillmann et al. 2003).

Since songbirds also need both hemispheres for song production, future research will provide us insight into the interhemispheric coordination for processing complex vocal sequences. How the two hemispheres cooperate is not yet clear. Could the two hemispheres be working in a way to periodically switch dominance? For example, dolphins use this strategy to sleep and swim at the same time by keeping one hemisphere active for hours, then fading away while the other hemisphere takes over (Mukhametov et al. 1988; Rattenborg et al. 2000; Sekiguchi et al. 2006).

Furthermore, it has been reported that music shares similar hierarchical phrase
structures with symbolic languages (Lerdahl 2001; Patel 2003). Understanding the detailed neural mechanisms underlying the processing of such syntactic information remains a challenge. By recording neuronal activity in the human brain, recent research has demonstrated that different levels of linguistic information are indeed sequentially processed within Broca’s area (Sahin et al. 2009). Neural recordings in Broca’s area revealed distinct neuronal activity for lexical (~200 ms), grammatical (~320 ms), and phonological (~450 ms) processing. The results were identical for nouns and verbs. However, such rare invasive experiments on human subjects are extremely difficult to reproduce and extend. In birdsong, syllable elements are also sequentially organized. The search for these unitary song elements and the study of their neural representations will help us understand the neural mechanisms for sequential processing of sequence behaviors.

This thesis is organized to present my research on using systematic perturbation techniques (acute electrical and noninvasive visual stimulations) of the bird’s brain. I have studied the two topics introduced above: (1) understanding universal dynamics underlying the birdsong process across both hemispheres; and (2) determining unitary birdsong elements and the transitions among them. I hope to find evidence supporting the analogy between the neural sequentiality of speech and birdsong.
Chapter 2: Brief review

2.1 Neural basis and terminology of birdsong

Zebra finches learn their song by imitating the songs of older male members (tutors) of their own species (Immelman 1969). Song learning is accomplished by modifying vocal output with the help of auditory feedback until a close match to a memorized song template is generated (Konishi 1965). If the juvenile birds are isolated from their tutors, they develop highly abnormal songs. Song learning starts with a stage that has been likened to human infant babbling (Aronov et al. 2008) called “subsong.” During this stage, highly variable, low-amplitude sounds are produced in a non-communicatory context, often while the juvenile seems to doze. The sounds of subsong provide the raw material from which imitation emerges. As these imitations become recognizable, they are referred to as “plastic song.” Once the imitations are perfected, the song becomes less and less variable. The stereotyped song is complete by the time the sexually mature bird is ready to start to defend a territory and woo a mate. After this point, the song will not change significantly. Figure 2.1 shows the schematic representation of song learning and production timeline.

Figure 2.1. Schematic timeline representation of song learning and production.

The standard terminology used to describe the components of adult zebra finch song is shown in Figure 2.2. The song of adult zebra finches consists of a series of “syllables.” Each syllable is a continuous trace on a sound spectrogram (sound frequency spectrum vs.
time), lasts 50-120 ms, and is separated from adjacent syllables by either silence or a rapid change in frequency modulation. Several syllables (typically 2-8) are produced in a fixed temporal order and constitute a “motif.” When an adult male zebra finch sings a “song bout”, the bird repeats the motif several times preceded by repetitions of a short “introductory note.” Other than singing, the bird can also arbitrarily utter single calls which can be either innate or learned from his tutor (David S 2004). In contrast to songs, which are used in territorial and reproductive contexts, calls are used for general communication on a daily basis. Distinct calls send particular messages, e.g., food begging, alarm, presence of food, contact, etc.

![Figure 2.2. Illustration of song components on spectrogram.](image)

Various sound features include: sound amplitude, amplitude modulation (AM), frequency modulation (FM), Wiener entropy, and pitch [see (Tchernichovski et al. 2000) for details]. These features and the temporal order of syllable of the song motifs are very stereotyped (see Figure 2.3). This is a good reason for using perturbation techniques to investigate how systematic stimulation can quantitatively perturb stereotyped behavior. By examining the perturbation effects, we will be able to infer the underlying neural mechanisms that generate and control the behavior.
Figure 2.3. Stack plot of sound features computed from non-perturbed song motifs.
Each upper panel shows the spectrogram of a bird’s song motif. The middle panel shows the stack plot of each type of sound features computed for 100 randomly selected song motifs aligned to a song detection.
point indicated by the white arrows. The lower panel displays a trace of sound features that include: (A) sound amplitude, (B) amplitude modulation (AM), (C) frequency modulation (FM), (D) Wiener entropy, and (E) pitch.

The acquisition and production of learned song is carried out by a set of discrete brain nuclei, referred to as the “song system” (Immelman 1969; Nottebohm and Arnold 1976; Nottebohm et al. 1976). This system has two main branches: the posterior descending pathway (PDP), which is necessary for both the acquisition and production of learned song, and the anterior forebrain pathway (AFP), which is only necessary for song acquisition (see Figure 2.4).

Figure 2.4. Diagram of a sagittal section of a generalized songbird brain.
Blue: posterior descending pathway (PDP); Red and Orange: anterior forebrain pathway (AFP); Grey: auditory pathway; Green: thalamic projections to PDP; Yellow: dopaminergic inputs to AFP; Purple: respiratory circuitry in the brain stem with projections to PDP and thalamus. (by Heather Williams, http://www.williams.edu/Biology/Faculty_Staff/hwilliams/Finches/circuits.html)

In mammals, the PDP is homologous to a motor pathway that starts in the cerebral cortex and descends through the brain stem (Nottebohm and Arnold 1976), while the AFP is homologous to a cortical pathway through the basal ganglia and thalamus (Nottebohm and Arnold 1976; Bottjer and Johnson 1997; Luo and Perkel 1999). Several of the telencephalic nuclei that participate in the production and acquisition of learned
song are small in nestlings, before the onset of song development. Nucleus volume, cell number, cell size, and inter-neuron connections grow during the subsequent weeks and months. As a result of these changes, many components of the circuits responsible for the acquisition and production of learned song are formed during the period when song first develops.

The main cerebral brain areas for vocal production are the robust nucleus of the arcopallium (RA), HVC (used as a proper name), and the lateral magnocellular nucleus of the anterior nidopallium (LMAN) which forms the output of the avian basal-ganglia pathway (Nottebohm and Arnold 1976). It is known that lesions of HVC result in more severe song distortion than the RA (Nottebohm and Arnold 1976). Stimulation of HVC during song interrupted and reset the motor program. This did not happen if the stimulating electrode was in RA (Vu et al. 1994). This hierarchical relation between HVC and RA was confirmed by recording from HVC and RA while the bird sang (Yu and Margoliash 1996). HVC neurons fired very sparsely and at narrowly defined times: each neuron fired during the same six-millisecond window while the bird produced its song (Hahnloser et al. 2002). For some songbirds whose songs change seasonally, their HVC neurons can be replaced due to neurogenesis when they modify their songs (Nottebohm 2002). These results implied that these HVC neurons—and the PDP of which they are a part—code the learned pattern of song. Although anatomically HVC is located at the highest hierarchical level in the song system, HVC mainly receives projections from the nucleus interface of the nidopallium (NIf) in the PDP and from the nucleus medial magnocellular nucleus of the anterior neostriatum (MMAN) in the AFP.

NIf exhibits both sensory and premotor activity, but whether it is necessary for both auditory and premotor processing in its target, HVC, is still unknown. Cardin et al. showed that bilateral NIf lesions result in long-term loss of HVC auditory activity but do not impair song production (Cardin et al. 2005). Spontaneous activity patterns in the nucleus RA and in HVC of the sleeping songbird resemble premotor patterns in these areas observed during singing (Dave and Margoliash 2000). Hahnloser et al. previously reported that the role of NIf in shaping these sleep-related activity patterns (Hahnloser et al. 2006). They found that inactivating NIf with a GABA-A agonist muscimol lead to transient abolishment of premotor-like bursting activity in HVC neurons. In combination with the previous findings by Cardin et al., it appears that somewhat different neural mechanisms underlie the generation of song sequences and song-replay in HVC of adult birds (Cardin et al. 2005).

So, what is the role of the AFP in song learning? It was known that the AFP was necessary for the acquisition of song, but not for the production of learned song (Bottjer
The nucleus MMAN is a small cortical region that has been implicated in song behavior based on its neuronal projection to the HVC. Foster et al. (Foster and Bottjer 2001) investigated the function of MMAN on song by making lesions of this brain region in juvenile male zebra finches during the period of vocal learning (40-50 days of age) and in adult males that were producing stable song (>90 days of age). Birds which obtained MMAN lesions as juveniles produced highly abnormal and poor quality songs as adults. Although the overall song quality of birds which obtained MMAN lesions as adults was not highly disrupted or abnormal, the postoperative song behavior of these birds was discernibly different due to slight increases in variability of vocal production, particularly at the onset of singing. These results demonstrate that MMAN plays an important role in vocal production during the sensory period for song learning and is also important for consistent initiation and stereotyped production of adult song behavior.

It was also known that the typically variable song of juvenile songbirds became very stereotyped after bilateral lesions of the LMAN (part of the AFP). From this, it was inferred that LMAN plays a crucial role in fostering the circuit plasticity necessary for learning (Scharff and Nottebohm 1991). Although the mechanism for this effect remained unknown for some time, Ölveczky et al. showed that the LMAN neurons that project to RA fire in a quasi-random pattern when variable song is produced in juvenile birds (Ölveczky et al. 2005). Thus, while the HVC→RA projection code for the learned song, the LMAN→RA projection code for the jitter that induces the variability in motor output necessary for the imitation of a model. This jitter is presumably imposed on the firing of the same RA neurons that receive the more orderly output from HVC. When the LMAN neurons are silent (or absent), the HVC→RA pathway produces a stereotyped pattern; when the LMAN→RA neurons are firing, song is more variable (Aronov et al. 2008). Finally, LMAN has been found to form NMDA synapses onto RA and disruptions of these synaptic connections during song learning by infusing LMAN or RA with NMDA blockers results in the birds developing highly abnormal songs (Aamodt et al. 1996; Basham et al. 1996). Based on these facts, the song-control pathway, from HVC in PDP through AFP and back to RA in PDP is thus involved in both song learning and song production.

Song-related neural activity in premotor brain areas is precisely coordinated across hemispheres, because both hemispheres contribute to the production of one unique and highly stereotyped song. This precise coordination is illustrated by the strong synchronization of multiunit activity in left and right HVC during singing (Schmidt 2003). One peculiarity of this system is that the right and left sides of the brain can operate, to
some extent, independently. Each half is responsible for a different part of the song (Schmidt 2008). In birds such as the canary, the chaffinch, and the white-crowned sparrow, a majority of the sounds of song are produced by the left half of the syrinx, which is under the control of the left hemisphere via uncrossed pathways. This phenomenon has been referred to as “left hypoglossal” or “left hemispheric” dominance (Nottebohm 1977). However, zebra finches are “right hemispheric” dominant (Williams et al. 1992).

2.2 Electrical stimulation for birdsong study

In the past decade, electrical stimulation experiments have established casual links between the activity of groups of neurons and cognitive functions (Salzman et al. 1990; Afraz et al. 2006). Based on the hypothesis that electrically stimulating the neural components of the song motor program during singing should alter the subsequent singing pattern, in 1994 Vu et al. first did electrical stimulation experiments in singing birds (Vu et al. 1994). They found that by unilaterally stimulating the forebrain area RA in singing birds, ongoing syllables were distorted without changing the order or timing of ensuing syllables. However, stimulating forebrain area HVC, which projects directly to RA, often suspended ongoing motifs and thus caused a temporal shift of succeeding motifs. Vu et al. showed that the time of motif suspension was locked to the HVC stimulation times (Vu et al. 1994). These findings supported the idea that HVC functions as a central pattern generator (CPG) generating commands for song and that subsequent components of the pathway merely execute these commands. Using deafened birds, they also showed that such song perturbation was not mediated by auditory feedback because stimulating HVC still altered song patterning of the deafened birds. The observation of unilateral forebrain perturbation of this bilaterally organized singing behavior suggests that non-auditory feedback pathways to the forebrain exist to coordinate between the two cerebral hemispheres during singing.

In 1998, Vu et al addressed the issue of such interhemispheric coordination of premotor neural activities during singing (Vu et al. 1998). They unilaterally recorded neural activity in the forebrain song nucleus HVC during singing and perturbed the neural activity in the contralateral HVC with electrical stimulation. Although it is known that there is no direct connection between the right and left forebrain song nuclei, they found that perturbing the activity in one HVC at some time during a song led to a readjustment of activity in the contralateral HVC with mean latency of ~36 ms. This adjustment consisted of a true resetting of the temporal pattern of activity suppression
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overlaid on an unaltered pattern of premotor activity. Their results strongly suggest that the song motor program during singing is continuously monitored and that there exists an active mechanism for re-synchronizing the outputs from the two hemispheres whenever their temporal patterns differ significantly. The location of this “synchronization program” and how it operates, however, remain unknown.

In 2003, Schmidt et al. (Schmidt 2003) reported experiments that demonstrated synchronized neural activity in HVC across two hemispheres. They speculated that the only anatomical pathways that could mediate synchronization and signaling between the HVC nuclei must pass through the brainstem, implying that feedback from the brainstem can in fact influence activity in HVC. In light of this, in 2005, Ashmore et al. reexamined the functional roles of the various song motor nuclei, again using electrical stimulation (Ashmore et al. 2005). In addition to HVC and RA, they stimulated brainstem structures and also recorded air sac pressure. The stimulation effects were divided into two classes: syllable level (including syllable distortion and truncation) and song level (including song restart or motif termination).

Unilateral HVC stimulation evoked both song- and syllable-level effects, often in tandem. Syllable truncation in particular was almost always accompanied by song-level effects. The average latency from HVC stimulation to syllable truncation was ~75 ms (Ashmore et al. 2005). However, the respiratory patterns measured by air sac pressure could be modified in less than 20 ms (Ashmore et al. 2005). This suggests that syllable truncation might not simply be the result of a disruption in the stimulated HVC that was transmitted directly to brainstem circuits; but rather that the extra time may reflect a requirement for disruption of the contralateral HVC before the full behavioral effects are manifest. Comparing their results to (Vu et al. 1998), the authors note that the mean latency to resetting activity in contralateral HVC (36 ms) was longer than that to the first measurable changes in air sac pressure but was much shorter than the latency to syllable truncation (Ashmore et al. 2005), consistent with propagation down to the brainstem and back to the contralateral HVC. In contrast to the results of (Vu et al. 1994), Ashmore et al. reported that even weak (15 μA) unilateral stimulation in RA could trigger song-level effects. In fact, low-intensity stimulation of RA was just as effective as stimulation in HVC (Ashmore et al. 2005). It is possible that the song-level effects of RA stimulation are attributable to antidromic disturbance of HVC activity rather than orthodromic effects propagating through normal feedback pathways. However, the authors go on to show that stimulation of medullary nucleus paraambigualis (PAm) within the brainstem respiratory network could also trigger song-level effects in addition to the expected syllable-level effects (Ashmore et al. 2005). Because HVC does not project directly to the brainstem,
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the song-level effects cannot be explained by antidromic disturbance of this putative CPG. In contrast, stimulation of the tracheosynrinal portion of the hypoglossal nucleus, which lacks ascending feedback projections did not evoke song-level effects (Ashmore et al. 2005). These data demonstrated that feedback from the brainstem can affect the motor program that drives song production.

However, this is just one possibility; these data do not exclude the hypothesis that HVC plays a privileged role in generating the song motor pattern. In vitro studies have shown that HVC can generate rhythmic activity patterns in isolation (Solis and Perkel 2005), and brainstem feedback may only coordinate activity patterns generated by HVC. Indeed there exist other feedback pathways relayed by the midbrain nucleus DM and the thalamic nucleus Uva. These bypass the brainstem and may be alternative candidates that serve the interhemispheric coordination of the HVC activity (see Chapter 4).

In 2005, Kao et al. (Kao et al. 2005) focused on the anterior forebrain pathway (AFP) that is necessary for song learning and adult vocal plasticity but not for song production (Bottjer et al. 1984; Scharff and Nottebohm 1991; Heather and Neil 1999; Brainard and Doupe 2000). They showed that song-triggered electrical stimulation in the output nucleus of the AFP induced acute and specific changes in the fundamental frequency and the sound amplitude of the song. In Figure 1C and 1D in (Kao et al. 2005), unilateral electrical stimulation in LMAN during certain syllables induced a downward shift of fundamental frequency of certain syllables and a decrease of sound amplitude. This finding demonstrates the capacity of the AFP to direct real-time control in song. In combination with anatomical evidence that MMAN in the AFP receives bilateral projections from the thalamic nucleus dorsomedialis posterior (DMP), it seems that the AFP may be partially involved in the interhemispheric coordination of song production. The stimulation experiments on anesthetized birds described in Chapter 4 provide some evidence to support this hypothesis.

2.3 Brain lateralization and interhemispheric switching

All vertebrates develop morphologically with several symmetric pair-wise parts. For example, humans have two hands, two feet, two ears, and two eyes, etc. The vertebrate brain is also symmetrically organized with two similar cerebral hemispheres. However considering each half of brain as a mirror image of its contralateral counterpart is an oversimplification. Careful anatomical comparison of selected structures and their connections reveal asymmetries across sides (Toga and Thompson 2003). These asymmetries are not only restricted to anatomical features but also include extensive
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functional differences between cerebral hemispheres (Sperry 1974). The existence of asymmetries implies that the two hemispheres are not only two massively redundant networks but are rather functionally specialized entities that work synergistically to coordinate the behavior of the organism. Hemispheric specialization, or lateralization, was originally thought to be a unique human characteristic, but it appears to be a general property among vertebrate brains. There are many examples of hemispheric lateralization, such as the specialization of the right hemisphere to process and store visual inputs that are important for memory imprinting in chicks (Gunturkun 1997) as well as different auditory processing characteristics in the left and right auditory forebrain of songbirds (Cynx et al. 1992; George et al. 2005). Many species, such as amphibians, reptiles, fish, and birds, also show functional lateralization in the periphery, suggesting hemispheric specialization (Vallortigara et al. 1999). Many migratory birds, for example, sense the direction of the magnetic field using only their right eye (Wiltschko et al. 2002). In songbirds, the lateralized mechanism allows the syrinx to generate abundant acoustic diversity, including independent sounds simultaneously produced by the two sides (an example also described in Chapter 6), and rapid switching of song production from side to side (Doupe and Kuhl 1999; Schmidt 2008).

Classical literature revealed that specific behaviors were lateralized to a single hemisphere (Geschwind 1970). The new view, however, is that individual hemispheres are not necessarily dedicated to a single behavior but are instead specialized for specific features of those behaviors (Serrien et al. 2006). In the context of language, for example, both hemispheres are involved in some aspect of speech processing and production, even though the left hemisphere might appear more dominant. The left hemisphere is, for example, more specialized for lexical and syntactical aspects of language, while the right hemisphere is more sensitive to emotional features of speech (Friederici and Alter 2004). There is supportive evidence that properties of the auditory cortex parallel these specializations. The left auditory cortex is more sensitive to fast temporal features of sound that are necessary for phonemic-level speech processing. In contrast, the right auditory cortex is sensitive to the slow rhythmic patterns in sounds that are associated with prosody, rhythm, stress, and intonation of speech (Giraud et al. 2007). Even at the level of speech production, a behavior originally thought to be controlled exclusively by the left hemisphere, there is now clear evidence that both hemispheres participate in the phonation process (Terao et al. 2001). Many behaviors require the recruitment of specializations from each hemisphere. In the case of language, syntax and prosody need to be combined to produce coherent speech patterns. Given the known hemispheric specializations for language, this combination requires the coordinated engagement of
both hemispheres. It has also been reported that music perception are engaged in both hemispheres (Koelsch et al. 2002; Tillmann et al. 2003).

This engagement may be simultaneous, i.e., both hemispheres are active at the same time in a compensatory manner. For example, the navigational process of a homing pigeon makes different use of left and right hippocampus formation (HF). The intact pigeon demonstrated preferential use of the sun compass with left HF in locating the goal, but the left HF lesioned pigeon demonstrated no ability to locate the goal by the sun compass but an ability to use the feature cues with the intact right HF (Gagliardo et al. 2005). Alternatively, engagement may alternate, i.e., hemispheric control switches from one hemisphere to the other. For example, transcranial magnetic stimulation (TMS) in parietal lobes of both hemispheres during mental imagery tasks, revealed interhemispheric compensatory interactions (Sack et al. 2005). The left parietal lobe was predominant in generating mental images, whereas the right parietal lobe was specialized in the spatial comparison of the imagined content. Furthermore, in case of a repetitive TMS-induced left parietal lesion, the right parietal cortex could immediately compensate such a left parietal disruption by taking over the specific function of the left hemisphere.

Although behaviorally, it has been reported that songbirds can rapidly switch syrinx from side to side for song production, the neurophysiological concept of hemispheric switching during the production of a single goal-directed behavior remains unclear. However, switching in hemispheric activation for perception has been observed in a number of animals, including humans (Miller et al. 2000). Hemispheric switching can be observed under a variety of conditions and on multiple time scales. At the slow end of the spectrum, switching back and forth between hemispheres can be observed during sleep in many aquatic mammals and birds (Rattenborg et al. 2000). Sleep-like activity as measured by EEG recording will occur in a single hemisphere for several minutes before switching over to the contralateral hemisphere (Lyamin et al. 2008).

At slightly faster rates, interhemispheric switching can be observed during eye movement in animals that can independently control each eye, such as chameleons and sand lances. These animals never generate saccades in both eyes at the same time, but instead generate a run of saccades in one eye before switching to the other eye after 10 to 20 seconds. Because the visual pathways in these animals are entirely crossed, the pattern of eye movements implies hemispheric switching at that same rate (Pettigrew et al. 1999). At the fast end of the spectrum, interhemispheric switching can occur at a rate of about 1 Hz during specific perceptual rivalry tasks in humans (Sheppard and Pettigrew 2006). Interestingly, the rate of interhemispheric switching during these tasks is not fixed because it can vary significantly with mood shifts and is much slower in subjects with
manic depression (Miller et al. 2003).

Although only a few examples of interhemispheric switching have been discovered so far, the wide range of conditions as described above, over which such switching can be observed suggests that it might be a general mechanism of brain function in bilaterally organized brain systems. The adaptive advantage of switching between hemispheres might be apparent in sleeping animals, because it allows animals the possibility of having one hemisphere in an awake state at all times. But the role that such switching plays in motor production is still unclear and the primary unanswered question is why a brain has evolved to switch rapidly between hemispheres. Functional specialization is found throughout the nervous system but requires communication among many different areas of the brain to result in cohesive activity. Interhemispheric switching may provide a compelling example, and may very well elucidate more general principles of brain function and the production of temporally complex behaviors.
“Just as the electron does not have a precise position and motion, I believe that consciousness has no location.”
George Wald (1906-1997)

Chapter 3: Rapid interhemispheric switching dynamics during song production in a zebra finch

To generate complex bilateral motor patterns such as those underlying birdsong, neural activity must be highly coordinated across the two cerebral hemispheres. However, it remains largely elusive how this coordination is achieved, given that interhemispheric communication between song-control areas in the avian cerebrum is restricted to projections received from bilaterally connected areas in the mid- and hindbrain. By electrically stimulating cerebral premotor areas in zebra finches, we find that behavioural effectiveness of stimulation switches rapidly between hemispheres. In time intervals in which stimulation in one hemisphere tends to distort songs, stimulation in the other hemisphere is mostly ineffective, revealing an idiosyncratic form of motor dominance that bounces back and forth between hemispheres like a virtual ping-pong ball. The intervals of lateralized effectiveness are broadly distributed, and the behavioural effectiveness patterns from the left and right hemispheres are found to dominantly cohere with the sound features of amplitude modulation (AM) and frequency modulation (FM), respectively. Furthermore, for a bird which likes to improvise a rendition of different number of his learned calls immediately following its song motifs, the interhemispheric switching pattern was also presented during these song-induced calls, but just not observed during single calls without singing. Such interhemispheric switching could be an important dynamical aspect of neural coordination that may be a legacy inherited from simpler pattern generator circuits in our wormlike ancestors.

3.1 Introduction

Owing to its complexity and high precision, birdsong has provided an important animal model for studies of motor control. Adult zebra finch songs are formed by repetitions of a highly stereotyped motif that is composed of 2-8 syllables and is acquired from a tutor during a critical sensori-motor period (Immelman 1969). Because the stereotypy of
birdsong is sustained after removal of auditory feedback, birdsong has been thought to be organized by a “central motor program.” (Konishi 1965; Konishi 1985; Vu et al. 1994)

A useful method to probe the functional roles of premotor brain areas is electrical stimulation. In general, electrical stimulation during motor production leads to specific behavioural distortions that depend on the location of stimulation electrodes (Schiller and Stryker 1972; Fried et al. 1991) as well as on the stimulation time (or phase) within ongoing motor patterns (Miller et al. 1977; Terao et al. 2001; Haridas and Zehr 2003; Kao et al. 2005). For example, in LMAN, which is involved in modulating birdsong by social context (Scharff and Nottebohm 1991; Hessler and Doupe 1999), unilateral electrical stimulation induces small transient effects on sound amplitude or sound pitch, depending on the precise stimulation time within the ongoing song motif (Kao et al. 2005). In HVC, which generates adult song by means of ultra-sparsely firing ‘clockwork’ neurons (Hahnloser et al. 2002; Nottebohm 2002), unilateral electrical stimulation also leads to transient song degradations such as syllable distortions and syllable truncations (Ashmore et al. 2005). More importantly, both LMAN and HVC stimulation sometimes induce non-transient effects such as song stoppings or early song restarts (Vu et al. 1994). During such restarting events caused by HVC stimulation, ongoing premotor activity in the contralateral HVC is reset within a few tens of milliseconds (Vu et al. 1998). Given that there are no direct interhemispheric connections between cerebral song-control areas, interhemispheric synchronization and song resetting must rely on common inputs to the song-control system from bilaterally connected mid- and hindbrain areas (Foster et al. 1997; Vates et al. 1997; Reinke and Wild 1998; Striedter and Vu 1998; Schmidt et al. 2004; Ashmore et al. 2007) (See Figure 3.1).

To further explore the dependence of song distortions on stimulation time, we chronically implanted bipolar stimulation electrodes in HVC in adult male zebra finches. We trained an artificial neural network to reliably detect the earliest possible note in a song motif in real time and stimulated either right or left HVC with a brief 0.4 ms biphasic (0.2 ms/phase) current pulse at random time lags after detection. We frequently interleaved stimulation trials with catch trials during which no stimulation was delivered. We also explored the temporally modulated effectiveness of LMAN stimulation by using suitable multi-pulse current trains delivered to bipolar stimulation electrodes implanted in LMAN (Kao et al. 2005).
3.2 Results

In line with earlier work, we found that unilateral HVC and LMAN stimulation distorted songs at the levels of song syllables and song motifs (Figure 3.2A) (Vu et al. 1994; Vu et al. 1998; Ashmore et al. 2005; Kao et al. 2005). By definition, syllable-level effects were restricted to the stimulated syllable or the subsequent syllable and consisted of either syllable distortions or syllable truncations. On the other hand, motif-level effects were manifest in longer time windows after stimulation and consisted of sudden song stopping or early motif restarts (see methods for exact definition of effects). The prevalence of syllable and motif-level distortions caused by HVC and LMAN stimulation is reported for all birds in Table 3.1.
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When hundreds of stimulated motifs were reordered by stimulation time, a temporal contiguity of stimulation effects became apparent in which nearby stimulation times led to qualitatively similar song distortions (Figure 3.2B). Hence, song distortions were not random, but were often deterministically linked with stimulation time, possibly caused by strong perturbation of stereotyped premotor activity.

Table 3.1. Percentages of HVC and LMAN stimulations that lead to syllable-level effects (truncations and distortions) and to motif-level effects (stoppings and restarts).

<table>
<thead>
<tr>
<th>Bird Name</th>
<th>Truncation HVC-R</th>
<th>Truncation LMAN-R</th>
<th>Distortion HVC-R</th>
<th>Distortion LMAN-R</th>
<th>Restart HVC-R</th>
<th>Restart LMAN-R</th>
<th>Stopping HVC-R</th>
<th>Stopping LMAN-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>p17k_p2</td>
<td>19.0%</td>
<td>10.7%</td>
<td>43.4%</td>
<td>34.0%</td>
<td>48.1%</td>
<td>42.3%</td>
<td>12.7%</td>
<td>14.2%</td>
</tr>
<tr>
<td>p13_l6</td>
<td>12.2%</td>
<td>5.8%</td>
<td>42.4%</td>
<td>35.9%</td>
<td>53.3%</td>
<td>50.3%</td>
<td>3.6%</td>
<td>3.2%</td>
</tr>
<tr>
<td>red13</td>
<td>14.8%</td>
<td>13.5%</td>
<td>26.9%</td>
<td>21.4%</td>
<td>5.8%</td>
<td>3.0%</td>
<td>42.8%</td>
<td>36.8%</td>
</tr>
<tr>
<td>yellow13</td>
<td>3.2%</td>
<td>0.7%</td>
<td>26.9%</td>
<td>25.7%</td>
<td>21.9%</td>
<td>22.5%</td>
<td>9.7%</td>
<td>15.3%</td>
</tr>
<tr>
<td>Distortion Long</td>
<td>18.3%</td>
<td>11.1%</td>
<td>16.4%</td>
<td>3.5%</td>
<td>5.4%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distortion Short</td>
<td>13.0%</td>
<td>13.5%</td>
<td>26.9%</td>
<td>21.4%</td>
<td>5.8%</td>
<td>3.0%</td>
<td>42.8%</td>
<td>36.8%</td>
</tr>
<tr>
<td>Restart HVC-R</td>
<td>12.7%</td>
<td>14.2%</td>
<td>14.8%</td>
<td>13.5%</td>
<td>26.9%</td>
<td>21.4%</td>
<td>5.8%</td>
<td>3.0%</td>
</tr>
<tr>
<td>Restart LMAN-R</td>
<td>3.6%</td>
<td>3.2%</td>
<td>42.8%</td>
<td>36.8%</td>
<td>5.4%</td>
<td>3.6%</td>
<td>3.2%</td>
<td>42.8%</td>
</tr>
<tr>
<td>Stopping HVC-R</td>
<td>42.8%</td>
<td>36.8%</td>
<td>5.4%</td>
<td>3.6%</td>
<td>3.2%</td>
<td>42.8%</td>
<td>36.8%</td>
<td>5.4%</td>
</tr>
<tr>
<td>Stopping LMAN-R</td>
<td>3.6%</td>
<td>3.2%</td>
<td>42.8%</td>
<td>36.8%</td>
<td>5.4%</td>
<td>3.6%</td>
<td>3.2%</td>
<td>42.8%</td>
</tr>
</tbody>
</table>

‘R’ and ‘L’ refer to the right and left hemisphere, respectively; ‘Short’ and ‘Long’ refer to the inter-electrode distance in HVC-implanted birds; and, the row labelled ‘StDev’ contains the standard deviations across birds.

A more detailed analysis revealed that song distortions most frequently occurred on the syllable level within several tens of milliseconds after stimulation. The probability of sound-amplitude distortions sharply increased 20 ms after stimulation, peaked roughly 50 ms after stimulation, and decayed thereafter (Figure 3.2C). This sharp rise agrees with measurements of air sac pressure deviations, the average onset of which lags HVC stimulation by 15-20 ms (Ashmore et al. 2005), whereas the late decay suggests that some perturbations of neural activity were transient and affected only a subpopulation of neurons.

Interestingly, on a fine time scale, not all distortions were locked to stimulation time. We occasionally observed syllables that were truncated not with a fixed delay to stimulation, but during a fixed time point with respect to the unperturbed motif (Figure 3.2D and 3.2E). In these cases, stimulation needed to occur within some time interval before a particular note in order to truncate that note, revealing that the motor program exhibits time points of high perturbation sensitivity. And, more interestingly, sometimes stimulation effects such as early motif restarts occurred neither after a fixed latency to
stimulation nor at a fixed time point of the unperturbed motif, but at some intermediate time (Figure 3.2D), further demonstrating nonlinear timing aspects of the song motor program.

**Figure 3.2. HVC stimulation leads to song distortions at the syllable and the motif levels.**

(A) Spectrogram of an adult male zebra finch song bout (top) with song motifs indicated by horizontal arrows. Below are a zoom into a normal (nonstimulated) song motif and examples of syllable-level stimulation effects (truncation and distortion, indicated by asterisks), and examples of motif-level effects (stopping and restart, with the restarting point indicated by an asterisk). (B) Top: Spectrogram of the song motif in (A). Bottom: Sound-amplitude stack plot depicting stimulation effects observed over the course of a day. Trials are ordered by stimulation time, marked by white dots. The top 200 traces depict nonstimulated catch trials, revealing the high stereotypy of song motifs. The pink arrows indicate corresponding stimulation trials in (A).
Stimulation effects display a contiguity, as revealed for example by nearby stimulation times that lead to persistent syllable truncations and early motif restarts (top pink arrow). (C) Effects on sound amplitudes started 20 ms after HVC stimulation and peaked after about 50 ms. Shown is the histogram of time bins with amplitude effects as function of latency to stimulation, normalized to the peak and averaged over stimulation sites (average over n=20 HVC stimulation sites in 10 birds). On average, the peak effectiveness occurred after about 50 ms, well within the EE window. (D) An example in which HVC stimulation over a broad temporal range leads to identical syllable truncation times. In the unperturbed spectrogram (catch trial, top) we marked the normal offset time of a selected syllable with the magenta vertical dashed line. Below are song spectrograms with identical syllable truncation times (pink vertical dotted lines) for both early and late stimulation (stimulation times are marked by white vertical lines). The sound amplitude stack plot (between the stimulation examples) reveals that all syllable truncations (pink dots, shown only for intermediate stimulation times) are vertically aligned, irrespective of stimulation time (white ramp dots, left). Note also that syllable truncations were followed by an unusual syllable that was never observed at this location during catch trials. The onsets of this appended syllable are marked by white vertical dashed lines in the ‘early’ and ‘late’ stimulation examples and by the cloud of white dots in the stack plot (shown for intermediate stimulation times). An F-test revealed that the onset times of this appended syllable had the same variance when measured relative to song detection time and relative to stimulation time (p=0.1). Hence, the timing of this syllable was neither fixed to stimulation time, nor to song time. (E) A stimulation example in a different bird in which syllable truncations (pink dots) tended to occur during a particular note rather than at a fixed time lag after stimulation. Same legend as in (D). (F) LEs increased with stimulation current. The LE curve associated with the stack plot in (B) is depicted by the blue curve (150 μA, single pulse). LE curves for higher and lower stimulation currents are also shown (brown and black curves). With increasing current, more stimulation times lead to LEs. The lower panel illustrates the computation of effect curves in this bird. The red line indicates stimulation time; black rasters indicate 3.9-ms time bins in which stimulation-related sound amplitudes were significantly different from baseline; the green lines delimit the time windows in which EEs and LEs were read out. Note that the stack plot in (F) is not perfectly (horizontally) aligned with that in (B) because of randomness of stimulation times [wiggly white line in (B)].

We automated the inspection of song distortions by analysis of sound amplitudes. We were mostly interested in motif-level effects because these seemed to arise from wide-spread and irreversible perturbation of premotor activity. For each stimulation time, we computed a late-effect (LE) value, defined as the fraction of 3.9 ms time bins in a 78-312 ms window after stimulation in which sound amplitudes were significantly different from amplitudes recorded during catch trials (see Methods). LE curves as a function of stimulation time had many sharp peaks that corresponded to different motif-level effects, separated by troughs in which stimulation was rather ineffective (on
Chapter 3: Rapid interhemispheric switching dynamics during song production in a zebra finch

the motif level). When we increased the stimulation currents, the set of effective stimulation times grew, as revealed by LE peaks that grew in height and width (Figure 3.2F). At the extreme of very high currents on the order of 0.5 - 1 mA, birds always stopped singing and significant LEs were seen for all stimulation times (n=3 birds, data not shown). In this study, our experimental strategy was to rapidly tune stimulation currents in order to observe highly modulated LE curves with coexistence of very large and close-to zero values, a task that typically was achieved within two days. At the current intensities chosen, LE curves displayed diverse peaks (the mean peak width at the effectiveness threshold was 20 ms, median 8 ms, range 4 to 160 ms, n=20 HVC stimulation sites in 10 birds). This wide range of peak widths in LE curves indicates that HVC stimulation perturbed neural activity on multiple time scales. The strong modulation of LE curves suggests rapid waxing and waning of the ipsilateral HVC drive, raising the question about modulation in the contralateral hemisphere.

To probe evidence of lateralized stimulation effectiveness, we implanted birds with stimulation electrodes in both left and right HVC and performed unilateral stimulation in randomly chosen hemispheres and at random time lags after note detection. After sorting all trials recorded over 1-3 days by hemisphere and stimulation time, a remarkable complementarity became apparent: for most stimulation times, stimulation effects were seen either for right- or left-side stimulation but not for both (Figure 3.3A; see Figure S1 for all birds used in our study). LE curves associated with left and right HVC stimulation were strongly modulated, but in an alternating fashion. We quantified the interhemispheric complementarity of stimulation effectiveness by the correlation coefficient (CC, see Methods) between right and left LE curves, and found that negative correlations prevailed (average CC -0.36, range -0.68 to -0.01, n=10 birds). To assess the significance of these anti-correlations, in three birds we implanted two pairs of stimulation electrodes in right HVC (in a cross arrangement). By running the same experimental protocol on the two ipsilateral stimulation sites in HVC, we found that CCs between corresponding LE curves were positive (average CC 0.36, range 0.25 to 0.46, n=3 birds), illustrating that the dependence of stimulation effects on electrode position within HVC is weak and demonstrating that the anti-correlation of stimulation effectiveness in bilateral stimulation experiments was highly significant. Moreover, in two birds we implanted stimulation electrodes in right HVC and right LMAN and also found positive CCs between corresponding LE curves (0.65 and 0.51, Figure 3.3B). The CCs in all birds are depicted in Figure 3C (see Table 3.2 for additional characterizations of the complementarity of right and left LE curves). We interpret this complementarity as
evidence that interhemispheric motor coordination involves temporally alternating neural mechanisms for song vocalization.

<table>
<thead>
<tr>
<th>Bird Name</th>
<th>% LE HVC-R</th>
<th>% LE LMAN-R</th>
<th>% Overlap</th>
<th>% Non-overlap</th>
<th>CC</th>
</tr>
</thead>
<tbody>
<tr>
<td>g17k_p2</td>
<td>33.7%</td>
<td>34.7%</td>
<td>27.8%</td>
<td>12.7%</td>
<td>0.51</td>
</tr>
<tr>
<td>p18k_s6</td>
<td>74.6%</td>
<td>69.7%</td>
<td>66.1%</td>
<td>12.0%</td>
<td>0.65</td>
</tr>
<tr>
<td>red13</td>
<td>37.3%</td>
<td>31.6%</td>
<td>19.8%</td>
<td>29.3%</td>
<td>0.46</td>
</tr>
<tr>
<td>yellow13</td>
<td>58.5%</td>
<td>39.8%</td>
<td>35.0%</td>
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<td>0.38</td>
</tr>
<tr>
<td>yellow18</td>
<td>20.0%</td>
<td>38.2%</td>
<td>16.0%</td>
<td>26.2%</td>
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**Table 3.2. Complementarity of stimulation effectiveness in two hemispheres.**

Reported are the percentages of stimulation times that were associated with significant late effects (LEs) on sound amplitudes (first two columns). Also reported are the percentages of stimulation times associated with overlapping/non-overlapping LEs at the two stimulation sites (third and second-last columns), and the cross-correlations (CCs) of the associated LE curves (last column). Percentages for each bird are indicated relative to the set of all stimulation times. Labels are as defined in the legend of Table 3.1.
Figure 3.3. Interhemispheric switching of stimulation effectiveness.

(A) Across hemispheres, effective HVC stimulation times are complementary. Shown are sound amplitude stack plots for left HVC stimulation (left) and for right HVC stimulation (right). Corresponding LE curves
(filled) are plotted against each other in the center to illustrate the alternating stimulation effectiveness. The correlation coefficient between left and right LE curves in this bird is -0.62. Inset (long black arrow): A zoom into the harmonic stack reveals no obvious relation between spectral syllable composition and right/left LE curves shown below: The transition from left to right effectiveness (right arrow) occurs in the middle of the harmonic stack where there is no apparent song change. (B) Within hemispheres, effective stimulation times are strongly overlapping. Shown are sound amplitude stack plots for HVC stimulation in right hemisphere (HVC-R) and for ipsilateral LMAN stimulation (LMAN-R). Corresponding LE curves (filled) are plotted against each other in the center to illustrate the alternating stimulation effectiveness. The correlation coefficient between left and right LE curves in this bird is 0.65. (C) Bar plot, showing the average correlation coefficient of LE curves for stimulation in opposite hemispheres (top) and in the same hemispheres (bottom). Symbols indicate correlation coefficients in individual birds: for unilateral HVC-HVC stimulation using two electrode pairs (crosses), for unilateral HVC-LMAN stimulation (diamonds), and for bilateral HVC-HVC stimulation (circles).

Communicating songbirds such as a zebra finch produces calls as well as songs, and some of these are learned (David S 2004). Some birds tend to improvise several repetitions of a learned call, appending it to his song. Since the call is not part of the bird’s normal song, how the interhemispheric switching pattern can be observed during the song-accompanied calls? We examined one particular bird “b8r7” whose non-singing calls (single call) was likely learned from his tutor “p2r5” (see Figure 3.4). This bird tended to improvise several repetitions of a call and appended it to his song motifs (song call). The bird usually exclusively uttered the single calls in a communicating manner when not singing (David S 2004). It is still not clear whether the single call and the song call are generated from the same neural mechanism although the sound similarity scores (see Methods) between them are very high. The mean similarity scores for different types of call pairs: “son’s single call vs. tutor’s single call,” “son’s song call vs. tutor’s single call,” “son’s single call vs. son’s song call,” and “tutor’s single calls vs. tutor’s single call” are 72.2±4.53%, 70.0±6.23%, 74.9±5.91% and 74.9%±10.3%, respectively. Since all these mean similarity scores are equally high (paired Student’s t-test, p>0.05), we can infer that the son’s single call and song call may be of the same identity and were learned from the tutor.
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Figure 3.4. The call of “b8r7” was learned from its tutor.

(A) Spectrogram and acoustic wave of their calls. A “Single Call” is the call uttered when the bird is not singing while a “Song Call” is the call uttered immediately following the song motif. (B) The mean similarity scores for different types of call pairs are equally high and not significantly different (paired Student’s t-test, n=100 pairs for each type, p>0.05). Error bars mean one standard deviation.

The HVC stimulation results as shown in Figure 3.5A clearly demonstrate that when the bird is engaged in the singing condition, interhemispheric switching governed the repetitive call renditions immediately following the song motifs, because the LE curves for the two hemispheres are highly complementary (anti-correlated, CC=-0.49). For right-HVC stimulation in the early part of the first call (see the dashed window on the right stack plot in Figure 3.5A), the calls were clearly truncated at the times locked to the stimulation times. These truncation points were determined by the times of the sound-energy offset (energy down crossed the baseline, see Methods) of the first call which was not followed by a second call. The mean truncation latency for the stimulation times within the first call (marked by the dashed window in Figure 3.5A) following the song motif is 63.3±12.7 ms. Over three days of experiments, the distributions of truncation latency are very stable as shown in Figure 3.5B [Kolmogorov-Smirnov (KS) test, p>0.05], and their mean values are all mutually not significantly different (paired Student’s t-test, p>0.05).
Figure 3.5. Interhemispheric switching of stimulation effectiveness during the song calls.

(A) The correlation coefficient between left and right LE curves in this bird is -0.49 that demonstrates the complementary effective HVC stimulation times across hemispheres. The black dashed window depicts the range where the right-HVC stimulation induced the truncations in the first call following a song motif. (B)
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The distribution of truncation latencies occurred within the dashed windows in (A). The mean latencies for each of the three experimental days are 66.3 ms, 60.9 ms and 58.5 ms, respectively.

However, when the bird was uttering communicating calls exclusive of being immediately adjacent to song motifs (calls not in song bouts, say the non-singing condition), can the same interhemispheric switching stimulation effectiveness still be observed? To answer this question, we analyzed the cases where the stimulations only randomly occurred in the single calls interleaved between song bouts (these song bouts have been analyzed and exhibited interhemispheric switching effectiveness as shown in Figure 3.5A). It is very striking that these single calls can be truncated at almost random times, with no correlation to the stimulation times, which is very different from the case as demonstrated in Figure 3.5. Figure 3.6A demonstrates that the stimulation effects lack any significant interhemispheric complementary patterning. Figure 3.6B shows that the stimulation induced single-call truncations have a broadly distributed temporal latency which is statistically different from those for song calls (Kolmogorov-Smirnov test, p<0.01).

This would suggest that the motor program used to generate single calls under various putative non-singing conditions may temporally deviate from a more precise rhythm necessarily required for singing. Furthermore, these results suggest that such patterning can stem from a switching dynamics which specifically attributes to a bird’s singing behaviour. And when the bird is doing something other than singing, his similar calling vocalization is not governed by such interhemispheric switching coordination. Although it is well known that birdsongs are mainly used by birds for mating purpose, the difference between what the calls and songs mean for a bird is still not clear. But when we make parallels between birdsong and human language or music abilities, we can check whether the intimate cooperation between the two hemispheres makes use of specialized processing power in each hemisphere for specific features of a vocal behaviour. Therefore, we were interested in determining whether the events at which stimulation effectiveness switched from one hemisphere to the other were locked to salient song features and whether the resulting switching intervals obeyed any regularity.
Figure 3.6. Weak and much random stimulation effectiveness during single calls.

(A) The correlation coefficient between left and right LE curves in this bird is -0.08 that demonstrates very weak complementary HVC stimulation effectiveness across hemispheres. (B) The distributions of truncation latencies occurred within the single calls are much broadened than the song calls. An asterisk indicates significance for the pair of latency distributions by paired KS test.
Visually, the effectiveness of electrical stimuli appeared to switch several times from one hemisphere to the other within a song motif, but often there was no obvious relationship between the discrete switching events and the sound spectrum produced at these times (inset of Figure 3.3A). When we assessed the events at which the effectiveness of electrical stimuli switched from one hemisphere to the other in terms of onsets of contralateral effectiveness (LE values larger than baseline), the mean switching interval was 35 ms (median 28 ms, range 4 to 150 ms). By contrast, when the switching events were defined by joint occurrence of ipsilateral ineffectiveness and onsets of contralateral effectiveness, the mean interval was 64 ms (median 44 ms, range 4 to 240 ms). Hence, on average, stimulus effectiveness switched back and forth between hemispheres within a few tens of milliseconds. However, our estimates of lateralized effectiveness and switching intervals must be interpreted with caution because of the aforementioned dependence of LE-peak widths on stimulus current, implying that switching intervals depend (non-trivially) on stimulus current. Nevertheless, because we found broadly distributed switching intervals both across all birds and within single birds, there is little evidence of periodicity in this interhemispheric switching process.

We further explored whether effective stimuli and their lateralization were related to specific sound features. Zebra finches mostly expire during syllables and inspire during syllable gaps (Goller and Daley 2001). Both expiratory and inspiratory nuclei in the brainstem project bilaterally and therefore may be involved in controlling effectiveness switching. Because we did not measure bronchial air flow, here we inferred respiratory patterns from sound pitch curves using the simplified assumption that zero pitch during syllable gaps corresponded to inspiration and nonzero pitch to expiration. We defined a rhythm curve as being equal to one during expiration and zero during inspiration. There was no significant coherence between this rhythm curve and either the right or left LE curves (see Methods). These results were unchanged when we defined expiratory patterns in terms of pitch values in the limited range 20-5000 Hz (thereby assuming that some high-pitched notes are generated during inspiration). Similarly, there was no significant coherence between right/left LE curves and each of the following: pitch curves (see Methods), syllable onset curves, and syllable offset curves (the latter were binary curves in which a pulse of variable width was set at the transitions between inspiration and expiration as assessed by the rhythm curve).

Although the evident for a consistent relationship between stimulation effectiveness and simple sound features is rather weak, we further examined its coherence with more profound sound features such as amplitude modulation (AM) and frequency modulation (FM), sound amplitude and Wiener entropy (see Figure 2.2 and (Tchernichovski et al.
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2000)). In an analogue telephone system, AM is the oldest method of transmitting human voice electronically, and about 30 years after the invention of AM, FM was also invented for radio systems to transmit information. These two paradigms can also be used to encode information in vocal signals (Zeng et al. 2005). Briefly, for sound-feature computation, the AM measure captures temporal changes in the amplitude envelope of sounds. It is simply the overall time-derivative power across all frequencies within a range (Tchernichovski et al. 2000). The unit of AM measure is “1/ms.” FM measure is estimated based on time and frequency derivatives across frequencies. It is defined as the angular component of squared time and frequency derivatives across frequencies (Tchernichovski et al. 2000). Unit of FM measure is “degree.” Wiener entropy is a measure of uncertainty that can be applied to sounds (Ho et al. 1998). It is a unitless number on a scale of 0–1 such that white noise has an entropy value of 1 while a pure tone (completely ordered) has an entropy value of 0. We used the Matlab (Mathworks, USA) function deriv.m (Tchernichovski et al. 2000) to compute these sound features and analyzed the coherence (see Methods) between these features and LE curves from 23 birds.

In Figure 3.7A, the coherence (blue curve) for left LE curves and AM of songs has two pronounced peaks in the frequency interval between ~5 and ~25 Hz, reaching the coherence value > 0.04 (p<0.01). Although we also see a short frequency interval between ~18 and ~22 Hz for significant coherence between right LE curves and AM of songs, the total coherent frequency interval (9.60 Hz) for the “left-LE” case (blue coherence curve) is larger than the total 5.41 Hz in the “right-LE” case (red coherence curve). Because LE curves characterize temporal changes of “susceptibility” of hemispheric activation for vocal production, we can define LE values as an alternative “firing rate” of a putative giant hemispheric neuron. Therefore the larger coherent frequency interval in the “left-LE” case can infer that left hemisphere may be more dominant in processing amplitude modulation of birdsong. The AM measure actually characterizes the degree of temporal changes of sound amplitudes. For the case of sound amplitudes (in unit of decibel), Figure 3.7B also shows a very similar coherence trend as the case of AM coherence as shown in Figure 3.7A.

In contrast to the left hemisphere which is more “AM sensitive,” the right hemisphere seems to be more dominant in processing FM of birdsong, because the total coherent frequency intervals (11.21 Hz) for the “right-LE” case is larger than the total 5.92 Hz in the “left-LE” case (see Figure 3.7C). Furthermore, the right hemisphere also seems to specialize for songs organized in terms of Wiener entropy which captures the uncertainty (also known as bits of informational complexity (Todd 2006)) of sound.
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Figure 3.7D shows that there only exists significant coherence (see red curve) between right LE curves and Wiener entropy of songs in the frequency intervals between ~18 to ~30 Hz.

Figure 3.7. Coherence between left/right LE curves and different sound features.

Each blue or red solid curve represents the coherence between each sound features (AM, FM, sound amplitude or Wiener entropy) and left or right LE curves, respectively. Data are combined for 28 right-hemispheric cases and 19 left-hemispheric cases from 23 birds in total. Dashed lines represent the $p=0.01$ confidence interval for the null hypothesis i.e., that the sound feature is independent of LE curves (see Methods). Thick blue or red bars represent the significant frequency intervals where the coherence between a sound feature and a left or right LE curve, respectively exceeds the confidence interval for the null hypothesis. (A) - (B) are coherence between LE curves and sound features: AM, sound amplitude, FM, and Wiener entropy, respectively.
Notice that our conclusions were reached from just a few seconds of effect-curve data (23 birds) and that it would be worthwhile to re-examine the relationship between stimulation effectiveness and song features in the future provided a larger body of interhemispheric stimulation data will be available.

3.3 Discussion

We have demonstrated an interhemispheric switching process for vocal production. In this process, the motor program exhibits perturbation sensitivity that rapidly alternates between hemispheres. Such alternation is surprising given that HVC activity is highly synchronized across hemispheres during singing and suggests that motor dominance rapidly switches back and forth between hemispheres. Possibly, the apparent alternation of dominance is related to birds’ ability to independently control the two halves of their vocal organ (Goller and Suthers 1995; Suthers et al. 2004). However, alternation is not synonymous with independent control as it represents a restriction on independence. If individual hemispheres are evolved to specialize for specific characteristics of a general behaviour, it will not be surprised that a brain uses switching strategy to integrate the hemispheric specialization for sensory or motor control.

It has been proposed that cortical asymmetries might have developed as a general solution to the need to optimize processing of the acoustic environment in both temporal and frequency domains, such that temporal resolution is better in left auditory cortical areas and spectral resolution is better in right auditory cortical areas (Zatorre et al. 2002). For the motor behavior, this study reported the first evidence that a songbird brain is also lateralized for specific features of its vocal output. We have found that the left-hemispheric stimulation effectiveness tended to be dominantly coherent with amplitude modulation of song, while the right-hemispheric stimulation effectiveness is more dominant in frequency modulation and Wiener entropy of songs. Our finding from the avian brains is surprisingly consistent to previous reports that in humans, left hemispheres were more susceptible to the signal envelope fluctuation (amplitude modulated) (Hiraumi et al. 2008; Okamoto et al. 2009), and left-hemisphere damage is associated with impairment in time analysis including the coding of envelope rate and shape, which may cause, in turn, speech intelligibility disorders (Lorenzi et al. 2000). A noise signal is usually amplitude modulated in nature. A recent study showed that human left hemispheres reflect a basic specialization contributing to the processing of complex auditory stimuli such as speech signals in noisy environments (Okamoto et al. 2007). This may be able to explain the origin of brain specialization for processing the
amplitude-modulated part of signals.

In contrast to left hemispheres, we also found that the right hemisphere is more coherent with frequency modulation of vocal signals, and moreover, is exclusively coherent to Wiener entropy of sounds. Our findings of such FM specialization in right hemispheres together with the AM specialization in left hemispheres also support the hypothesis from recent findings that spectral change is dominantly processed in the right hemisphere, whereas temporal change is dominantly processed in the left (Okamoto et al. 2009). Wiener entropy is a measure of the width and uniformity of the power spectrum that can characterize “uncertainty” of signals (Ho et al. 1998). In a previous study, avian call complexity was just measured in terms of uncertainty (bits of information), the diversity of potential messages conveyable by a signal system (Freeberg 2006). The biological significance of this study is that by manipulating the social group size, they found that individual Chickadees in larger groups used calls with greater complexity (uncertainty) than individuals in smaller groups. This result indicates that social complexity can influence communicative complexity in this species. Our finding of right-hemispheric dominance in the Wiener-entropy structure of sounds may provide a basic neural correlate in right hemispheres to process vocal complexity. Wiener entropy may also correlate with the dynamic state of the syringeal sound generator, which shifts between harmonic vibrations and chaotic states (Fee et al. 1998). Such transitions may be among the most primitive features of song production and imitation. This may be able to explain the right-hemisphere dominance for zebra finch song control (Williams et al. 1992) because during song development, imitation is actually a process in which its vocalizations shift from an initially more chaotic state into a structured final state, which can be correlated to some complexity measure such as the Wiener entropy.

Although we have found some evidence that a zebra-finch brain is lateralized for processing certain sound features, it is still difficult to ascertain which hemisphere is dominating at any time in this switching process. On the one hand, one could argue that stimulation should be more effective in a dominant hemisphere, because this hemisphere is being perturbed while generating a song in both syringeal halves. On the other hand, one could argue that stimulation should be less effective in a dominant hemisphere, because the perturbation is not strong enough to overrule the ongoing activity there. In the following we discuss the evidence for these two interpretations as well as for interpretations on whether stimulations perturb activity in local or in distributed networks.

From existing data we cannot infer whether the motor apparatus necessitates continuous and simultaneous drive from both cerebral hemispheres or not: adult birds do
not sing normally after unilateral RA lesions (Ashmore et al. 2007), but this data does not exclude the possibility that at any time the effective motor program resides in just a single hemisphere and bounces back and forth between hemispheres during singing. For example, if singing at all times is based on activity in just a single hemisphere and the drive provided by premotor activity in the other hemisphere is temporarily gated off, then we would conclude that the dominant hemisphere is the one in which low-intensity stimulation is effective. In this view, stimulation of the non-dominant hemisphere above a given current threshold would also be able to distort songs, because strong perturbations might ultimately find their way to the dominant side (pass the gate) where they could interrupt the ongoing motor program. However, if normal song production at all times requires simultaneous contributions from both hemispheres, then high stimulation effectiveness might be an attribute of the non-dominant hemisphere, because this hemisphere can be perturbed at lower stimulation currents. On the dominant side then, low-intensity stimulation would be corrected by redundant neural mechanisms that were not sufficiently perturbed by the stimulation.

Not only the dominance question is difficult to address, but it is similarly difficult to tell whether song disruptions were entirely due to perturbation of local ongoing HVC activity or of a larger distributed network. For example, one may speculate that the number of spiking RA-projecting HVC neurons might drift randomly up and down during the song motif (with some inertia). Such random drifts could be associated with a compensatory increase in the number of spiking neurons in the contralateral HVC and thus to alternation of dominance. A compensatory process could be regulated during song development (e.g., by neurogenesis (Paton and Nottebohm 1984) and programmed cell death), and therefore alternating dominance would not have to rely on real-time interhemispheric communication. According to this interpretation, LMAN and ipsilateral HVC stimulation lead to similar song distortions because LMAN stimulation perturbs RA-projecting HVC neurons for example via RA (Roberts et al. 2008). However, it is unclear why compensatory mechanisms would act across hemispheres but not within the same hemisphere. Furthermore, if the interhemispheric complementary stimulation effectiveness was due to such compensatory process during song development, stimulation during the learned calls under any behavioral context should reveal the same interhemispheric complementary patterns, but from the data of one bird, such patterning was only observed during song-accompanied calls, but not during the single calls without singing. This remarkable result suggests that the complementary process may stem from a switching dynamics specific for singing, but not just from static HVC-RA networks with different synaptic robustness developed across both hemispheres during song
development. The observations of interhemispheric synchronization of HVC activity and with some stimulation effects such as early song restarts would also suggest the dynamic nature of the interhemispheric discrepancy of stimulation effectiveness reported in this chapter.

The more likely scenario within which our observations can be explained is that LMAN and HVC stimulation induce similar song distortions because of widespread perturbation of sub-pallial structures via RA. Because we observed a wide range of switching intervals, we found little support for the idea that switching times are determined by fixed signal propagation times (for example as reverberating activity in closed synaptic loops) or by the fixed period of a simple pattern generator circuit. Rather, some switching events may arise from detection of specific premotor patterns in one hemisphere that are subsequently relayed to the contralateral hemisphere.

Interhemispheric switching processes in relation to motor production have been reported also in mammals, for example during the preparation of vocal production in humans, in which effectiveness of transcranial magnetic stimulation (TMS) of motor cortex alternates between hemispheres (Terao et al. 2001). Interhemispheric switching has also recently been shown to exist during perceptual rivalry, as evidenced by the hemispheric dependence of magnetic and calorimetric stimulation (Miller et al. 2000). Interhemispheric switching may thus be a fundamental mechanism by which sensory and motor-related activity is coordinated across hemispheres. In mammals, interhemispheric coordination seems to be mainly mediated by cortico-cortical projections (Brinkman and Kuypers 1973; Donchin et al. 1998). However, during saccadic eye movements of split-brain monkeys, activity in the two hemispheres has been shown to remain coordinated despite the lack of cerebral commissures, suggesting that sub-cortical pathways can subserve coordination also in the mammalian brain (Berman et al. 2005), and suggesting that similarities may exist between interhemispheric coordination in avian and mammalian brains.

Based on networks models, switching has been proposed to depend on competitive interactions (Hilgetag et al. 2001) mediated by inhibition (Moreno-Bote et al. 2007). It has been found that neuronal competition is necessary for refining neural circuits during development and may be important for selecting neurons during memory formation (Han et al. 2007). Evidence for interhemispheric inhibition has already been found in TMS studies of human motor cortex (Ferbert et al. 1992; Lo and Fook-Chong 2004). We speculate that interhemispheric switching in songbirds could also rely on inhibitory mechanisms. A possible function of such inhibition could be to suppress mirror-symmetrical movements which are thought to represent one of the default
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operation modes of bilateral motor systems (Swinnen 2002). In this sense, interhemispheric inhibition would coexist with more cooperative (excitatory) interactions between hemispheres. The excitatory mechanisms could be relayed by respiratory nuclei, known to generate mirror-symmetrical respiratory patterns (Goller and Suthers 1999), while the inhibitory gating mechanisms could be mediated by two possible pathways: the tonically spiking Uva-HVC projections (see also Figure 3.1) (Hahnloser et al. 2008), and HVC-RA and LMAN-RA projections feed-forward and feedback inhibiting RA projection neurons (Spiro et al. 1999).

One of the important finding of this study is that the bird required interhemispheric switching for song production but not for non-singing vocalization. This would suggest that the motor program for song vocalization may require a more precise rhythm (an idiosyncratic oscillation, see Chapter 5) for coordinating the process in both hemispheres such as the bilateral neural processing of music (Koelsch et al. 2002; Patel 2003; Tillmann et al. 2003). We have shown that the diverse oscillation periods of interhemispheric switching ranged from 4 to 160 ms, which are in the range of theta- and gamma-bands. Brain oscillations can control the timing of single-neuron activity in Humans (Jacobs et al. 2007), and a recent work just deciphered sequentially processed neural codes that can see through a rat’s “thoughts” self-generated in the hippocampus (Pastalkova et al. 2008). This could be a fundamental of known neural mechanisms for processing complex sequential information stored in the brain. For example, being distant from hippocampus, Broca’s area was also recently proven to sequentially process the complex linguistic sequence information (Sahin et al. 2009). There was a working hypothesis that recall of memory sequence is associated with theta and gamma oscillations (Jensen and Lisman 2005) and the theta responses are also involved in lexical-semantic retrieval during language processing (Bastiaansen et al. 2005). For song sequence production, whether songbirds use the same strategy as revealed from our interhemispheric switching oscillations within the theta- and gamma-bands will be an interesting unsolved question. One can expect an experiment to bilaterally measure local field potential (LFP) in HVC during unperturbed singing and thus analyze the correlation between the LFP oscillation patterns with LE curves. High correlation may just imply a positive answer to the question above.

In this study, the reported interhemispheric switching process is reminiscent of one of the most ancient and prominent “oscillation generators” with left-right alternating dynamics, which is locomotion. In vertebrates, locomotion is subserved by central pattern generators in the spinal cord which can display sustained rhythmic activity with left-right alterations even in in vitro preparations (Kiehn 2006). Because locomotion is much older
than birdsong on an evolutionary timescale, phase alternating neural circuits must have existed long before birds started to sing. Possibly, principles of limb coordination in locomotor circuits have been replicated by evolution for the more recent advent of birdsong. Some support for this idea comes from the conservation of bilateral projection patterns in brainstem nuclei of songbirds and non-songbirds (Wild et al. 1997). Vocal basis for acoustic communication among vertebrates has been proposed to evolve from an ancestrally shared hindbrain-spinal compartment already presented in the early fishes (Bass et al. 2008). It suggests that old brain circuits have just evolved to support new functions such as the birdsong.

### 3.4 Methods

**Subjects.** Adult (>90 days) male zebra finches (Taeniopygia guttata) were used for experiments. Birds were selected on the basis of singing frequency and song complexity, and were isolated in a sound-attenuating chamber. To maximize singing frequency, birds had visual contact to one or more female zebra finches through the glass door of the chamber. A total of 15 birds were used; data in one bird was discarded because HVC stimulation did not reliably produce motif-level effects. At the end of experiments, electrolytic lesions were performed at the stimulation sites by DC current injections (15 µA for 20 s), birds were killed by overdose of Nembutal, and stimulation sites were verified in histological brain sections. All procedures were approved by the Veterinary Office of the Kanton of Zurich.

**Real-Time Song Detector.** For all the chronic, song-triggered stimulation experiments, a real-time song detector was implemented to detect an early part of the song motif (in most cases the first or second syllable). Sounds were recorded and digitized at a sampling rate $f_s$ of 33 kHz. We generated a training set consisting of approximately 20-30 different renditions of the target syllable and a similar number of samples of each other syllable and sounds such as wing flaps, calls, and ambient noise. For this training set, we computed the time-frequency spectrogram $S(t,f)$ (temporal resolution $128/f_s=3.9$ ms, spectral resolution $f_s/256=129$ Hz), and transformed the spectrogram data into a set of vectors $X(t)$:

$$x_0(t) = \ln \sum_i S(t,f_i), \left( f_i = \frac{f_s}{256} \times i \right).$$


\[ x_k(t) = \ln \sum_{i=4(k-1)+1}^{4k} S(t, f_i), \quad (k = 1, 2, 3 \ldots 20), \]

\[ \tilde{x}(t) = (x_0(t), x_1(t), x_2(t), \ldots, x_{20}(t)), \]

\[ X(t) = (\tilde{x}(t), \tilde{x}(t-2\tau), \tilde{x}(t-4\tau), \ldots, \tilde{x}(t-10\tau)), \quad (\tau = \frac{128}{f_s}). \]

Each rendition \( n \) of the target syllable was aligned by the sound onset \( t_0^n \) (RMS threshold crossing). We manually selected a time offset \( \tau_0 \) to detect the target syllable. Then we defined the group of \( X(t_0^n + \tau_0) \) as the ‘positive’ detections and the group of all other \( X(t) \) as the ‘negative’ detections. We trained a two-layered feed-forward Artificial Neural Network (Matlab Neural Network Toolbox 4.0) with the ‘positive’ and ‘negative’ groups as network inputs, and the trained output values are 1 and -1, respectively. For real-time song detection, we took the set of parameters \( (w_1, w_2, b_1, \text{ and } b_2) \) obtained from the trained neural network and implemented a LabVIEW program (see Appendix A) to compute in real-time:

\[ Y(t) = Tansig(X(t) \cdot w_1 + b_1) \cdot w_2 + b_2, \quad \left( Tansig(u) = \frac{2}{1 + e^{-2u}} - 1 \right). \]

We defined that \( Y(t) \geq 0.2 \) gives a ‘positive’ detection, whereas \( Y(t) < 0.2 \) gives a ‘negative’ detection.

**Song-triggered microstimulation.** We delivered electrical stimuli with uniformly distributed probability over the time span of song motifs using custom written Labview software (National Instruments Corporation, Austin TX). With probability 0.35, detection triggered microstimulation at site A, with probability 0.35 at site B, and with probability 0.3 no stimulation was delivered (catch trials).

Electrodes were made of 50 \( \mu \)m stainless steel wire. Electrical stimuli in HVC consisted of a single 0.4 ms biphasic (0.2 ms/phase) current pulse of amplitude between 100 \( \mu \)A and 1 mA. In LMAN, electrical stimuli consisted of trains of 10 biphasic current pulses at 400 Hz (0.4 ms/phase; train duration 23.3 ms) and amplitudes in the range 10-100 \( \mu \)A. The current threshold at which single-pulse stimulation in LMAN induced motif-level effects (song suspensions) was high (typically > 1 mA). For this reason and to adhere to previous stimulation studies (Vu et al. 1994; Kao et al. 2005), we chose a multi-pulse paradigm in LMAN, in which we stimulated for 10 pulses at low currents (10 ~ 100 \( \mu \)A per pulse).
Chapter 3: Rapid interhemispheric switching dynamics during song production in a zebra finch

Analysis of syllable- and motif-level effects. We distinguished among different syllable and motif-level effects as follows:

**Syllable truncations:** First we measured baseline distributions of syllable lengths from data of selected catch trials (only complete motifs). Stimulated syllables were then classified as truncated if their duration was within the lowest percentile of the baseline distribution. We searched for truncations only in a time window up to 156 ms (corresponding to 40 time bins of 3.9 ms or 128 sound samples each) after stimulation.

**Syllable distortions:** In each time bin after note detection, we calculated the baseline distribution of sound amplitudes during selected catch trials (no spontaneous song stopping). We then counted the number of 3.9 ms time bins up to 78 ms post stimulation time in which the stimulation-related sound amplitude was significantly different from this baseline distribution (percentile p<0.025 or p>0.975). If this number was large enough (binomial test, alpha=0.05), then we classified this stimulation effect as a syllable distortion. Distortions and truncations were not mutually exclusive.

**Motif stoppings and restarts:** For each bird we chose a sound-amplitude threshold slightly above cage-noise level (we found that a threshold of 20% into the 1-99th percentile interval worked well for all birds). For all stimulation trials, under visual supervision, we then used this threshold to mark the offset time of every prematurely stopped motif and the successive restart time of the following note (independently of whether this note come from a song syllable, an introductory note, or a call). If the offset time fell into a window from 0 to 156 ms after stimulation and there was no restart until 312 ms, then we classified the stimulation effect as a stopping event. If, on the other hand, there was a restart after a premature offset within 312 ms after stimulation, then the stimulation effect was a restart. Hence, restarts and stoppings were mutually exclusive (however, song stoppings and syllable truncations were not).

**Automated analysis of stimulation effects in early and late-effect windows.** All songs (stimulation and catch trials) were aligned by detection time. For each stimulation site, we sorted the trials by stimulation time and grouped them into 9.75-ms sets with centers separated by 3.9 ms from each other. With a mean stimulation range of approximately 500 ms and typical detection of 800-2000 song motifs per day, we obtained roughly 3-8 stimulation trials per set per day. Typically, we collected a mean of 10-20 trials per set and then tested for each set whether the sound amplitudes in 3.9-ms bins after stimulation were different from amplitudes in matched time bins during catch trials using the Kolmogorov-Smirnov (KS) test (p<0.01). For each set we quantified the stimulation effect by the fraction of time bins in which significant differences were detected. Late
effect (LE) curves were based on bins ranging from 78 to 312 ms after stimulation (bins 21 to 80). Early effect curves were based on bins ranging from 0 to 78 ms after stimulation (bins 1 to 20). To assess the time scales of song perturbations, we computed the peak widths in LE curves at the effectiveness threshold, defined by the baseline LE value during catch trials (binomial test, p<0.01).

**Notes on robustness.** Our results did not depend critically on the EE and LE time windows in which syllable-level and motif-level effects were assessed. We chose the offset of the LE window (312 ms) as a compromise between being large enough to yield high sensitivity and small enough to not extend too far beyond the motif end where songs became highly variable. We set the onset of the LE window (or offset of the EE window, 78 ms) so as to exceed the peak time of stimulation effectiveness (Figure 2D), which was within 70 ms of stimulation (in agreement with previous reports (Vu et al. 1994)). Small changes in the LE window onsets (from 58.5 to 117 ms) and LE window offsets (from 234 to 390 ms) did not affect our findings of interhemispheric switching in any way.

By experimental design, our results were robust to variability in sound amplitudes caused by movements of the bird’s head relative to the microphone. That is, head-position variability must have had identical influences on sound amplitudes recorded during catch trials and during stimulation trials because we randomly chose all stimulation parameters right after each detection event (i.e., whether and where to stimulate, and the stimulation time). Hence, by design there were no correlations between head position and stimulation parameters.

**Similarity of effect curves.** We assessed the similarity between effect curves \(x\) and \(y\) associated with different stimulation sites by the (Pearson) correlation coefficient (CC) \(c = \frac{\text{Cov}(x,y)}{\sqrt{\text{Cov}(x,x)\text{Cov}(y,y)}}\) where \(\text{Cov}(x,y)\) is the covariance between \(x\) and \(y\). Because effect curves were non-negative, stimulation times for which both \(x\) and \(y\) were ineffective (compared to sound-amplitude variability before stimulation, binominal test at 99% significance level) imposed a bias toward positive correlations. To avoid this bias, we ignored bilaterally ineffective stimulation times when calculating the correlation coefficient (for LE curves these were 32% of all stimulation times). Note that our conclusions were unchanged when correlation coefficients (CCs) were calculated over the full set of stimulation times (thereby imposing a positive bias): the difference between average CCs in unilateral and bilateral stimulation experiments was highly significant in either case (p<0.001, Wilcoxon ranksum test).
Respiratory rhythm inferred from sound pitch. The relationship between right/left LE curves $y$ and the rhythm curve $z$ was investigated by the coherence:

$$C = |p(yz)|^2 / p(yy) / p(zz)$$

where $p(yz)$ is the cross-spectral density, and $p(yy)$ and $p(zz)$ are the power spectral densities of LE and rhythm curves, respectively. We chose the coherence function because its phase insensitivity allowed us to detect significant correlations irrespective of their time lag. We assessed the significance of coherence peaks by testing whether these exceeded two Jackknife estimates of standard deviation (corresponding to 95% confidence). The 10 Jackknives were defined by leaving out each of the 10 birds from the analysis.

Early-effect analysis. By visual inspection, stimulation effectiveness at the syllable-level showed weaker interhemispheric complementarity than those at the motif-level. Yet, early effect (EE) curves associated with bilateral stimulation (average CC $-0.13$, range $-0.68$ to $0.43$, $n=10$) showed significantly lower correlations ($p=0.019$, Wilcoxon rank sum test) than EE curves associated with unilateral stimulation (average CC $0.35$, range $0.04$ to $0.80$, $n=5$ birds). As before, to compute these CCs we only considered stimulation times that were associated with effectiveness in at least one hemisphere, thereby omitting 25% of stimulation times (compared to omission of 32% for LE curves). In conclusion, alternating effectiveness was seen most clearly for stimuli that disrupted normal singing, but also for stimuli that induced immediate amplitude distortions.

Pitch analysis. We investigated the possibility that pitch differences exist between times at which right and left HVC stimulation is effective. The coherence between the sound pitch curve and either right or left LE curves was not significant, neither when we considered the full pitch curve nor when we clamped the pitch curve to zero below either 2 or 5 kHz. Similarly, the median pitch during right-effective stimulation was not statistically different from the median pitch during left-effective stimulation (Wilcoxon ranksum test, $p=0.4$). We also tested whether pitch differences were seen at a particular time lag after effective stimulation times. We found that the median pitch 40 ms after left-effective stimulation was significantly higher than 40 ms after right-effective stimulation ($p=0.031$, $n=10$ birds). However, when we excluded any one of two particular birds from the analysis, then significance broke down ($p>0.1$). Significance also broke down when assessed using a shuffle predictor of pitch differences in songs of randomly shuffled syllables and gaps from different birds (Monte Carlo simulations, $p>0.05$).

Similarity Measurement. Similarity score for a pair of calls was quantified using the
Sound Analysis Pro software (ver. 2A.01) (Tchernichovski et al. 2000) that are the arithmetic product of the proportion (“similarity %”) of sound in one call for which there is a close correspondence in the other, and the pair comparisons of the acoustic features of identified song motifs 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 10 ms intervals and averaged over the duration of the entire song motif. Sounds are aligned in time so as to maximize the similarity, allowing for 5% time warping. For a comparison of two types of calls, asymmetric song similarity scores for 100 pairs (10×10) will be computed. Values are stated in the text as the mean ± SD.

**Calculation of Coherence.** To characterize the relation between stimulation effectiveness and the sound features of songs, we computed the coherence between mean-subtracted sound feature $S_{i,j}(t)$ and $E_{R/L,j}(t)$, where the symbolic subscript $i=AM, FM, AMP, or WE$ denote sound features of amplitude modulation, frequency modulation, sound amplitude, or Wiener entropy, respectively, the numeric subscript $j=bird$ number, and $R$ or $L$ denotes the right or left hemisphere, respectively. For the $j$th bird, $E_{R,j}(t)$ is the LE curve for right-hemisphere stimulation effectiveness while $E_{L,j}(t)$ is the LE curve for left-hemisphere stimulation effectiveness. $S_{i,j}(t)$ was computed using the Matlab code deriv.m with input arguments of sound signals and the sampling rate for sound recording (Tchernichovski et al. 2000). Direct spectral estimators of $S_{i,j}(t)$ and $E_{R/L,j}(t)$ were computed using the multi-taper method (MTM) (Thomson 1982) with time-bandwidth product $NW=7/2$ and $n_{FFT}=512$ points. MTM can provide estimates of both the singular components (i.e., the “lines”) and the continuous component (i.e., the “background”) of the spectrum. Once the tapers $w(t)$ and corresponding weights $u_k$ are computed for a chosen frequency bandwidth, the $k$th eigenspectrum $\tilde{S}_{i,j}(f)$ and $E_{R/L,j}^k(f)$ for the tapered version of $S_{i,j}(t)$ and $E_{R/L,j}(t)$ is the fast Fourier transform of $S_{i,j}(t) \cdot w_k(t)$ and $E_{R/L,j}(t) \cdot w_k(t)$, respectively. Data from different stimulation electrodes in different birds were regarded as different samples. The coherence between the $i$th sound feature and right/left LE curve was thus computed by summing cross-spectral products over the samples and tapers and normalizing (Thomson 1982):

$$C_{i,R/L}(f) = \frac{\left| \sum_{j=1}^{n_{R/L}} \sum_{k=1}^{(2+NW)} u_k \tilde{S}_{i,j}^k(f) \bar{E}_{R/L,j}^k(f) \right|^2}{\sum_{j=1}^{n_{R/L}} \sum_{k=1}^{(2+NW)} u_k \left| \tilde{S}_{i,j}^k(f) \right|^2 \sum_{j=1}^{n_{R/L}} \sum_{k=1}^{(2+NW)} u_k \left| \bar{E}_{R/L,j}^k(f) \right|^2}$$
(* denotes the complex conjugate; \( n_R = 28 \) stimulation electrodes in the right hemisphere of 23 birds, and \( n_L = 19 \) stimulation electrodes in the left hemisphere of 23 birds). The \((p\times100^{\text{th}})\) percentile confidence intervals for the null hypothesis (i.e., that sound features and LE curves are independent) of coherence \( C_{i,R/L}(f) \) are given by 
\[
1 - p^{1/(2+NW+n_{R/L}-1)}
\]
(Jarvis and Mitra 2001).
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The singing of a songbird is a complex motor behavior that requires precise coordination of neural activity across hemispheres. In Chapter 3, using electrical stimulation in the song nuclei HVC and LMAN, I reported the evidence of remarkable rapid switching dynamics between both brain hemispheres; however, the actual pathways for the cross-hemispheric dynamics remain unclear. In order to identify candidate pathways, here I report the putative connectivity revealed by performing brief microstimulation in HVC in anesthetized birds and simultaneously recording extracellularly in the contralateral forebrain song nuclei.

Multiunit hash activity was previously found to be evoked in HVC, NIf, MMAN and Uva by stimulating contralateral HVC. This suggests that NIf, MMAN and UVA might relay the interhemispheric transmission of neural signals for song production. In Chapter 2, I mentioned that the AFP nucleus MMAN might have a role in interhemispheric coordination via the thalamic nucleus DMP, a hypothesis that would be supported by the stimulation experiment mentioned above. In addition to DMP, Uva is the only other thalamic nucleus to relay bilateral ascending inputs from other song nuclei back to HVC, with either direct projection or indirect projection via NIf. Furthermore, it has been shown that the thalamic nucleus Uva receives information about ongoing motor instructions via respiratory and brainstem nuclei (Reinke and Wild 1998), and one of the Uva’s behavior-related functions is to control the temporal patterning of learned songs (Williams and Vicario 1993; Coleman and Vu 2005).

Using pharmacological manipulation, electrical stimulation, and extracellular recordings of Uva projection neurons in zebra finches, we studied the role of Uva in the generation of spontaneous activity and auditory responses in premotor area HVC and the downstream nucleus RA. In both awake and sleeping birds, we found that single HVC-projecting Uva spikes suppress spontaneous and
auditory-evoked bursts in HVC and RA neurons. Because chronic song-triggered HVC stimulation after bilateral NIf lesions still revealed stimulation effectiveness with interhemispheric switching patterns, NIf was ruled out as being a relay of interhemispheric coordination. As a result, the inhibitory Uva-HVC pathway could fulfill the requirements of an inhibitory mechanism which underlies the interhemispheric switching for birdsong production as discussed in Chapter 3.

4.1 Introduction

The bilateral neural activity which underlies birdsong production requires precise interhemispheric coordination - even though songbirds lack direct synaptic connections between song areas and across the two brain hemispheres. In Chapter 3, I have reported that during song production, interhemispheric dynamics exist which may have the function of interhemispheric coordination. However, the pathways that mediate the interhemispheric switching dynamics remain unclear.

In mammals, interhemispheric coordination seems to be mainly mediated by corticocortical projections (Brinkman and Kuypers 1973; Donchin et al. 1998). However, during saccadic eye movements of split-brain monkeys, activity in the two hemispheres has been shown to remain coordinated despite the lack of cerebral commissures, suggesting that subcortical pathways can subserve coordination in the mammalian brain (Berman et al. 2005). Similarities may exist between interhemispheric coordination in avian and mammalian brains.

At least three different anatomical pathways exist (Foster et al. 1997; Vates et al. 1997; Reinke and Wild 1998; Striedter and Vu 1998) that could provide the function of interhemispheric coordination as shown in Figure 4.1. Previous studies have used a combination of electrical microstimulation, stimulation-evoked vocalizations, targeted lesions, and paired recordings in different song-control nuclei to identify the bottom-up functional brainstem to forebrain pathways (the first pathway as shown in Figure 4.1B) (Ashmore et al. 2008). In this chapter, I used microstimulation and multiunit recordings to identify the remaining two pathways between the thalamic nuclei, Uva and DMP, and the AFP nucleus MMAN.

Based on networks models (Hilgetag et al. 2001; Moreno-Bote et al. 2007) and on the evidence found in TMS studies of human motor cortex (Ferbert et al. 1992; Lo and Fook-Chong 2004), interhemispheric switching has been thought to depend on competitive interactions mediated by inhibition, and it is reasonable to speculate that interhemispheric switching in songbirds could also rely on such inhibitory mechanisms.
Uva is the thalamic relay station in two of the pathways shown in Figure 4.1B and 4.1C. In songbirds, Uva provides the sole thalamic input to HVC, and it exhibits state-dependent responses to auditory presentation of the bird’s own song (BOS). Uva could gate auditory responses in HVC through a mechanism that involves local inhibition of HVC as well as withdrawal of auditory-evoked excitatory drive from NIf (Coleman et al. 2007).

To elucidate the role of Uva, we first performed HVC stimulation (described in Chapter 3) in a bird before and after NIf lesion to verify whether NIf cooperates with Uva in interhemispheric coordination. Then, in head-fixed zebra finches, we explored the involvement of Uva for the generation of spontaneous and auditory-evoked HVC and RA spike bursts. Using pharmacological and electrical manipulations, we examined whether Uva participates in the inhibitory mechanisms acting on HVC and RA.

Figure 4.1. Three candidate anatomical pathways that could serve interhemispheric coordination.
(A) The schematic of all bilaterally organized song-control areas in the zebra finch brain. Same as Figure 3.1
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and is used here as an overview. (B) - (D) simply dissect candidate pathways of interhemispheric coordination. (B) A pathway proposed in (Reinke and Wild 1998) that projects back to HVC via PAm, a structure known to contain inspiratory bulbospinal neurons (Wild 1997; Wild et al. 1998). Different stimulation sites and stimulation-evoked-activation areas are marked by purple and red. For example, RA can be activated by both stimulating contralateral PAm (marked by the red sign) and RA (marked by the purple sign). The stimulation-evoked activations in RA are then abstractly marked by filling red and purple corresponding to the stimulation in contralateral PAm and RA, respectively. Both paired stimulation-recording experiments for this pathway were firstly reported in (Ashmore et al. 2008). (C) A pathway proposed in (Striedter and Vu 1998) that projects back to HVC via the midbrain nucleus DM, a structure whose stimulation is known to generate unlearned vocalizations in songbirds (Vicario and Simpson 1995). The paired stimulation-recording experiments shown in both purple and orange are firstly reported in this chapter (see Figure 4.2A-E), whereas cases shown in red were firstly reported in (Ashmore et al. 2008). (D) A pathway proposed in (Foster et al. 1997; Vates et al. 1997) that projects back to HVC via the thalamic nucleus DMP instead of Uva. The paired stimulation-recording experiment shown in purple is firstly reported in this chapter (see Figure 4.2F), whereas the case shown in red were firstly reported in (Ashmore et al. 2008).

4.2 Results

The first pathway shown in Figure 4.1B was initially identified in previous studies using a combination of microstimulation, stimulation-evoked vocalizations, targeted lesions, and paired recordings (Ashmore et al. 2008). Their results suggested a crucial role for the respiratory brainstem in bilaterally coordinating forebrain song nuclei during song production. Here we used the same technique of microstimulation combined with paired multiunit recordings in the contralateral song nuclei to validate the other two pathways shown in Figure 4.1C and 4.1D.

Figure 4.2A-D shows poststimulus time histograms (PSTHs) of activation in right DM (latency=6.15±3.04 ms, n=2 DM in 2 birds), left Uva (latency=27.81±4.76 ms, n=5 Uva in 2 birds), left NIf (latency=17.28 ms, n=1), and left HVC (latency=26.94±4.76 ms, n=9 HVC in 3 birds) elicited by stimulating right HVC. All of the stimulation-evoked responses are significant by testing the measurement of post-to-baseline (see Methods) against the control-to-baseline (paired Student’s t-test, p<0.01).

The candidate pathway starting in HVC, passing through ipsilateral RA and DM, to contralateral Uva, and then to contralateral HVC (or via NIf, to contralateral HVC) forms a pathway which serves the function of interhemispheric coordination as illustrated in Figure 4.1C. Our results showing the stimulation-evoked responses along these song
control nuclei provide neurophysiological evidence that activation in HVC can drive neural activity across hemispheres and thus suggest that these activated song nuclei may be the sources which cause the interhemispheric switching patterns observed with HVC stimulation during vocal production.

Figure 4.2. Stimulation of forebrain and midbrain song nuclei in one hemisphere evokes activity in the contralateral hemisphere.

Stimulation occurred at time 0 and the stimulus artifact has been removed from the PSTHs. Stimulation is a single 0.2 ms wide, 400 µA monophasic pulse. (A) PSTH for stimulation in HVC and recording in ipsilateral DM. (B) - (D) PSTHs for stimulation in HVC and recording in contralateral Uva, NIf and HVC, respectively. (A) - (E) reveal stimulation responses in each song control nucleus along the pathway as shown in Figure 4.1C. (F) PSTH for stimulation in HVC and recording in contralateral MMAN that validate the pathway (Figure 4.1D) by showing that the stimulation in HVC can activate contralateral MMAN. All stimulation-evoked activity in (A) - (F) is significant by testing the peak-to-baseline ratio (see Methods) against the control-to-baseline (paired Student’s t-test, p<0.01).
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We also stimulated DM and recorded in contralateral HVC to confirm DM as being one of the relay nuclei in this pathway. Also plotted in PSTH, Figure 4.2E shows left HVC activation evoked by contralateral DM stimulation (latency=5.24±0.21 ms, n=2 HVC in 2 birds). In the third candidate pathway shown in Figure 4.1D, MMAN receives bilateral projection from thalamic nucleus DMP. A previous study has shown that pre-lesioning Uva and subsequently lesioning MMAN can further attenuate the contralateral RA stimulation response which was already weakened by lesion of Uva (Ashmore et al. 2008). Their study also showed that stimulating RA can activate contralateral MMAN (also see the illustration in Figure 4.1D). These results suggest that MMAN could also serve for the function of interhemispheric coordination. As shown in Figure 4.2F, we confirmed that MMAN can be driven by contralateral HVC activation (latency=10.51±0.68 ms, n=2 MMAN in 1 bird).

In the following I will reconcile the necessity of Uva and NIf in the first two candidate pathways. First of all, we tested whether NIf mediates the coordination by performing chronic HVC stimulation with bilateral pre- and post-lesion of NIf (n=2 birds). If NIf is important for coordinating the interhemispheric switching dynamics, lesions should result in deficits of the interhemispheric switching patterns. Figure 4.3 demonstrates the stimulation result with NIf pre- and post-lesioned bilaterally. The CC value between the left curves for NIf pre-lesion and NIf post-lesion is 0.39, while the CC value between the right curves for NIf pre-lesion and NIf post-lesion is 0.27. As the CC values for paired LE curves (NIf pre-lesion vs. NIf post-lesion) of each hemispheres are positive, and the corresponding curve shapes are also similar, these finding suggest that NIf is not necessary for interhemispheric coordination, which is in line with previous study that show that bilateral NIf lesions do not severely impair singing (Cardin et al. 2005). Therefore I will focus on Uva with a more detailed study about how Uva_{HVC} projection neurons regulate the activity of HVC and RA neurons.
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Figure 4.3. Bilateral lesions of NIf do not qualitatively impair the interhemispheric switching of stimulation effectiveness.

The sound stack plot together with the blue LE curves are from the bird with NIf intact. Across hemispheres, effective HVC stimulation times are complementary (CC=-0.43). The red LE curves are from the same bird after subsequent bilateral NIf lesion (CC=-0.1). The CC values between left LE curves for NIf pre-lesion and post-lesion (blue vs. red), and between right LE curves for NIf pre-lesion and post-lesion (blue vs. red) are 0.39 and 0.27, respectively that demonstrates no qualitative change of the interhemispheric switching of stimulation effectiveness resulting from bilateral NIf lesions.

Using antidromic stimulation in HVC and single-unit recordings in Uva of head-fixed zebra finches (see Methods), we were able to identify two classes of Uva projection neurons. HVC-projecting Uva neurons (Uva\(_{\text{HVC}}\) neurons) exhibited spike collisions at small time lags after spontaneous spikes during electrical stimulation in HVC (Figure 4.4Bi). We also performed HVC stimulation to identify NIf-projecting Uva neurons (Uva\(_{\text{NIf}}\) neurons). This eliminated possible confounds with Uva\(_{\text{HVC}}\) neurons that were also activated by NIf stimulation due to their axons passing in close proximity to
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NIf. Uva\textsubscript{NIf} neurons often responded to HVC stimulation, but did not display spike collisions (Figure 4.4Bii). Putative Uva\textsubscript{NIf} neurons exhibited spike collisions in response to low-amplitude NIf stimulation (Figure 4.4Biii).

We studied the relationships between Uva and HVC activity by recording from HVC interneurons (HVC\textsubscript{I} neurons). We did not record from HVC projection neurons, because the latter fire extremely sparsely during sleep. To explore the role of Uva in shaping activity along the motor pathway, we also recorded from RA neurons, which are known to be strongly driven by RA-projecting HVC neurons (Hahnloser et al. 2006). HVC is necessary for the generation of RA spike bursts but not of single spikes (Hahnloser et al. 2002). To avoid corruption of our analysis by RA single spikes, we often removed single spikes from RA spike trains prior to the analysis.

**Figure 4.4. Identification of Uva neurons using antidromic stimulation in HVC.**

(A) Schematic drawing of the song control pathway, showing the main premotor and motor areas and the experimental setup. The thalamic uveaform nucleus (Uva) projects to NIf and to HVC. HVC projects to the RA, which in turn innervates neurons in the midbrain nucleus DM and respiratory neurons in nucleus PAm in the medulla. These subpallial structures both project back to Uva, thus closing a respiratory–motor feedback loop (dashed arrows). We identified Uva neuron type by electrical stimulation in HVC and recorded from RA, HVC, NIf, and Uva neurons of the right hemisphere. (B) Electrical stimulation in HVC elicits spike responses in Uva neurons. Uva\textsubscript{HVC} neurons (i) exhibit spike collisions to HVC stimulation (200 \mu A) at small time lags after spontaneous spikes (arrows), but not at large time lags. Uva\textsubscript{NIf} neurons respond to 60 \mu A HVC stimulation (ii) and to 30 \mu A NIf stimulation (iii), but spike collisions are only observed for NIf stimulation, but not for HVC stimulation (ii and iii, lower traces).

HVC\textsubscript{I} neurons and RA neurons display strongly modulated firing patterns during sleep (Danoczy and Hahnloser 2005; Hahnloser et al. 2006). In paired recordings with Uva\textsubscript{HVC} neurons, we found that occasional periods of suppressed bursting in HVC\textsubscript{I} and RA neurons were often associated with periods of high tonic firing rates in Uva\textsubscript{HVC}.
neurons (Figure 4.5A, 4.2B, and 4.2D). Covariance functions between spikes in Uva_{HVC} and HVC_{1} neurons, as well as between spikes in Uva_{HVC} and burst-spikes in RA neurons, both displayed a large and wide negative peak (dip) that extended up to several seconds after Uva_{HVC} spikes (Figure 4.5C and 4.2E); in Uva_{HVC}-HVC_{1} pairs, a sharp and narrow dip was also seen at 1 ms of Uva_{HVC} spikes). Of course the existence of wide dips does not necessarily imply that single Uva_{HVC} spikes mediate long-lasting inhibition, because an important factor that contributes to dip width is the auto-covariance of Uva_{HVC} spike trains. One method of subtracting effects of auto-covariance is to inspect coherency functions rather than cross-covariance functions (see e.g. (Hahnloser and Fee 2007)). Indeed, when we evaluated coherency functions in Uva_{HVC}-HVC_{1} pairs, only the sharp dip at 1 ms after Uva_{HVC} spikes remained, suggesting that Uva-mediated inhibition is fast and of short duration. The average Uva_{HVC}-HVC_{1} covariance function also had a sharp positive peak located at -27 ms of Uva_{HVC} spikes (upper arrow in Figure 4.5C).

In RA neurons, no negative peak was seen, but a positive peak at -7 ms, implying that Uva_{HVC} spikes were often preceded first by HVC_{1} spikes, then by RA bursts. Note that the covariance results for RA neurons depended strongly on removal of single RA spikes. In contrast, results remained qualitatively unchanged when single HVC_{1} spikes were removed, presumably because most HVC_{1} neurons have low tonic firing rates (Hahnloser et al. 2006). Up to now our results suggest that single Uva_{HVC} spikes are inhibitory and driven by cerebral feedback via the midbrain, and that Uva_{HVC} burst are excitatory and drive cerebral bursts.

Multiunit recordings in anesthetized birds have revealed strong auditory responses in Uva to playback of the bird’s own song (BOS) (Coleman et al. 2007), suggesting that such Uva responses may be relayed to the cerebrum also during sleep. To that end, we investigated auditory responses of identified Uva projection neurons in sleeping birds.

Whereas RA cells burst in a more or less stereotypical manner in response to BOS playback, we found no obvious BOS-locked firing in many Uva_{HVC} cells (Figure 4.6A). In total, only 2/8 Uva_{HVC} cells (n=4 birds) had significantly increased average firing rates during BOS playback compared to equally sized time windows before playback (p<0.01). The number of BOS-responsive Uva_{HVC} cells did not change when either Uva_{HVC} single spikes or burst spikes were removed, suggesting that weak BOS responses are associated with both single spikes and spike bursts. Most interestingly, the average rate of RA burst spikes (the burst firing rate, BFR) in response to BOS playback was a smooth and decreasing function of the number of Uva_{HVC} spikes (Figure 4.6B). These results indicate graded suppressive effects of Uva_{HVC} spikes onto both spontaneous and auditory-evoked RA bursts.
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Figure 4.5. $Uva_{HVC}$ neuron spikes are negatively correlated with spikes in HVC interneurons ($HVC_1$ neurons) and bursts in RA neurons.

(A) Extracellular record of an $Uva_{HVC}$-HVC$_1$ pair recorded during sleep. (B) Instantaneous firing rate (IFR) functions of the neurons in a. Periods of high $Uva_{HVC}$ firing (top panel, thick horizontal bars) are coincident with periods of reduced HVC$_1$ bursting (bottom panel). $Uva_{HVC}$ bursts over 100 Hz have been truncated for better visibility of low firing rates. (C) The cross-covariance function of this neuron pair (top) and the average covariance function of n=7 $Uva_{HVC}$-HVC$_1$ pairs in 3 birds (bottom) both exhibit a broad dip extending up to 3 s of $Uva_{HVC}$ spikes and sharp positive and negative peaks close to zero time lag (arrows). To not smear over the sharp peaks, almost no smoothing was applied (4 ms wide Gaussian). (D) and (E) same as (B) and (C), but for RA instead of HVC$_1$ neurons (n=8 $Uva_{HVC}$-RA pairs). RA single spikes have been removed for the computation of covariance functions (see Methods). Smoothing was performed with a 20 ms wide Gaussian.
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HVC stimulation evokes activation in the contralateral forebrain song nuclei. The thalamus may gate interhemispheric coordination for birdsong production.

Figure 4.6. UvaHVC firing negatively correlates with RA bursting during BOS playback.

(A) Raster plots of simultaneously recorded UvaHVC and RA spike responses (tick marks) to playback of the bird’s own song (BOS). UvaHVC spikes are not obviously locked to the stimulus, but RA burst spikes are. (B) The burst firing rate in RA neurons to BOS playback is a monotonically decreasing function of the firing rate in UvaHVC neurons (mean ± s.e.m., n=8 UvaHVC-RA pairs).

We further explored whether the suppressive effects of UvaHVC single spikes were dominant by pharmacologically manipulating Uva activity. If UvaHVC single spikes are able to prevent HVC₁ neurons from bursting, then inactivating Uva should lead to increased HVC₁ burst firing rates. We reversibly silenced Uva by injecting the sodium-channel blocker lidocaine (4% lidocaine), or, to avoid accidental inactivation of fibres of passage, the inhibitory neurotransmitter GABA (250 mM GABA in 0.9% NaCl) into Uva of sleeping birds. Injected volumes were either 110 nl or 55 nl, corresponding to spheres of roughly 300 µm and 240 µm radius, both larger than the presumed volume of Uva. No qualitative differences between GABA and lidocaine injections were seen. In either case, HVC₁ bursting (n=3) and RA bursting (n=7) transiently increased for a few minutes following Uva inactivation (Figure 4.7A and 4.7B).

We analyzed the RA data in more detail and found that many aspects of RA neuron spike trains were identical before and after the injections: normalized interspike-interval histograms exhibited two identically located peaks corresponding to a regular firing mode and a bursting mode (Figure 4.7C). However, the relative heights of these peaks during Uva inactivation were such that RA neurons spent more time in the bursting mode when UvaHVC spikes were absent. Indeed, when we removed RA single spikes, we found that RA burst shapes were unchanged during Uva inactivation (as
assessed by the density of small interspike intervals), except that RA bursts occurred more often during inactivation (as assessed by the left-shift of the ISI peak corresponding to inter-burst intervals, inset Figure 4.7C).

Figure 4.7. Uva inactivation leads to burst-rate increases in RA neurons.

(A) IFR function of an RA neuron during sleep. During the time interval marked by the thick horizontal line, 115 nl lidocaine is injected into Uva, leading to a transient increase in RA bursting. (B) Bar plot summarizing the average increase in RA burst-firing rate in the 60 s interval after lidocaine injections (full circles) and GABA injections (open circles), compared to the 60 s interval before the injections (n=7 injections in 3 birds). The rightmost bar depicts the average burst-firing rate in the interval 400 – 460 s after the injections (recovery). (C) The average ISI probability density function (pdf) of RA neurons before the injection (full line) and during inactivation (dashed line). Both curves exhibit peaks at 5 and 55 ms (arrows), but of different relative amplitudes. Inset: ISI pdfs of RA burst spike trains reveal that burst shapes are unchanged during Uva inactivation (left-hand peaks are identical), but inter-burst intervals are shortened (tilted arrow).

RA neurons significantly increased their burst firing rates in response to BOS playback, both before and during Uva inactivation (Figure 4.8A). Despite this increase in playback-evoked responses, high temporal precision of individual RA bursts was
maintained during Uva inactivation (Figure 4.8B). Thus, Uva seems to have no major influence on the detailed timing of stimulus-evoked RA bursts. BOS-response enhancement was specific to Uva inactivation: Uva injections of vehicle (0.9% NaCl), and GABA injections 0.8 mm dorsal of Uva did not cause significant changes in RA bursting (n=2 birds each). In two birds, GABA injections 0.8 mm anterior of Uva caused strong suppression of RA responses to BOS playback; histological examination revealed fluorescent staining of the lateral part of nucleus ovoidalis, suggesting that the decrease of RA auditory responses was due to diminished auditory input, but not due to Uva inactivation.

Figure 4.8. Uva inactivation enhances RA burst responses to BOS playback.

(A) Bar plot showing that RA burst firing rates in response to BOS playback increase during Uva inactivation (n=4 steady GABA injections in 3 birds for a duration of 16 BOS stimuli each). RA neurons respond to BOS playback both before and during Uva inactivation (‘Spont’ bars indicate spontaneous RA BFRs measured during silent periods just before playback onsets).

(B) Raster plot of an RA spike train in response to BOS playback before and after GABA is steadily injected into Uva (GABA injection onset is indicated by the horizontal arrow). Uva inactivation slightly increases temporally locked RA burst responses (vertical arrow).

To test further whether Uva

HVC

spikes can cause suppression of sleep-related and auditory-evoked HVC

I

and RA bursts, we injected the excitatory neurotransmitter glutamate (in 0.9% NaCl) into Uva. When we pressure injected small volumes (23 nl) of 12 mM glutamate into Uva of sleeping birds, we observed no significant reduction in RA bursting (n=3 injections in 3 birds, p>0.01). At equal volume but higher concentration (23 nl of 50 – 200 mM glutamate), we observed robust suppression of auditory-evoked
and spontaneous RA bursts (Figure 4.9A). Suppression was transient and bursting recovered within 20 – 40 seconds. On average, 50 mM glutamate injections into Uva significantly reduced spontaneous and evoked RA burst-spike rates from 2.1 Hz to 0.2 Hz (20 second windows pre and post injection; Figure 4.9B). At higher concentration and injected volumes, HVC\textsubscript{1} neurons often dramatically increased their spike frequency (Figure 4.9C).

![Figure 4.9. Dose-dependent effects of glutamate injections into Uva.](image)

(A) IFR response of an RA neuron to BOS playback during sleep. During the time interval marked by the thick horizontal line, 23 nl of 50 mM glutamate is injected into Uva, leading to a transient suppression of BOS-locked bursting. (B) Bar plot summarizing the reduction in RA burst-firing rate in the 15 s interval after glutamate injections, as compared to the 15 s interval before the injection. The connected circles depict data from different injections (n=3 birds). (C) In this HVC\textsubscript{1} neuron BOS responses were transiently suppressed after 3 nl, 7 nl, and 14 nl injections of 1M glutamate into Uva.

In most song-control brain areas such as RA and HVC, neural activity is strongly gated depending on the behavioural state of birds such as sleep, wakefulness, and anaesthesia (Dave et al. 1998; Schmidt and Konishi 1998; Cardin and Schmidt 2004). Based on the negative correlations between Uva\textsubscript{HVC} spikes and RA bursts, we expected Uva\textsubscript{HVC} neurons to increase their firing when birds are woken from sleep. Indeed, when we aroused birds by brief air puffs — the awake state was assessed by open eyes and by
lack of bursts in RA neurons (Hahnloser et al. 2006) — \( \text{Uva}_{\text{HVC}} \) firing rates significantly increased from 12.8 to 27.3 Hz (burst firing rates in RA neurons significantly decreased from 2.9 to 0.02 Hz; Figure 4.10A and 4.10B). Interestingly, even though arousal led to increases in average \( \text{Uva}_{\text{HVC}} \) firing rates, average \( \text{Uva}_{\text{HVC}} \) burst firing rates decreased significantly from 0.49 to 0.10 Hz (Figure 4.10Biii).

Both the wake–sleep dependence of \( \text{Uva}_{\text{HVC}} \) firing and the suppressive action of \( \text{Uva}_{\text{HVC}} \) spikes onto RA bursts suggest that \( \text{Uva}_{\text{HVC}} \) spikes might be strongly implicated in mediating the behavioural-state dependence of bursting in RA. To test for this possibility, we aroused birds by air puffs following Uva inactivation. If Uva controls the behavioural-state dependence of RA activity, then RA bursting should be unaffected by arousal when Uva is silenced. If, instead a common neuromodulatory input to Uva and RA leads to behaviourally-mediated firing modulation in these areas, then RA bursting should be strongly suppressed, irrespective of Uva activity. We found that waking birds after large lidocaine or GABA injections into Uva significantly reduced bursting in RA, but did not completely abolish it (Figure 4.10C and 4.10D). Thus, it appears that \( \text{Uva}_{\text{HVC}} \) spiking is one part of the input required for RA to generate burst suppression associated with arousal.
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Figure 4.10. Uva\textsubscript{HVC} firing depends on the behavioural state.

(A) IFR plots of an Uva\textsubscript{HVC}–RA pair. When the bird is aroused by a light air puff (arrow), RA bursting stops, whereas Uva\textsubscript{HVC} tonic firing increases. (B) Bar plots summarizing firing changes related to arousal. Firing rates and burst firing rates were measured in equal time windows before and after the air puffs. The durations of these windows were set by the time during which the awake state could be verified in terms of open eyes. (Bi-Biii) Burst firing rates in RA (Bi) and Uva\textsubscript{HVC} (Biii) neurons drop significantly, whereas instantaneous firing rates in UVA\textsubscript{HVC} neurons (Bii) significantly increase. (C) Arousing birds by air puffs following lidocaine or GABA injections leads to partial suppression of RA bursting. (D) Bar plot summarizing the reduction in RA burst firing measured in 30 second windows before and after waking, both within 3 minutes of the injections (n=6 injections in 3 birds, lidocaine injections shown by full circles and GABA injections shown by open circles).
4.3 Discussion

Singing is a motor sequence behavior that requires precise control of timing. The ability of short perturbation in HVC to reset the entire song pattern suggests that the putative bilateral timing source requires input from HVC in order to generate the next precise timing signal in the sequence (Schmidt and Konishi 1998; Schmidt et al. 2004). However since HVC is not not directly connected across two hemispheres, it suggests that a network of bilaterally connected structures in the thalamus, midbrain, or brainstem is responsible for generating these timing signals (see also the illustration in Figure 4.1). It is therefore of great interest to understand the nature of the timing signal that reaches HVC.

As shown in Figure 4.1, HVC receives only projections from thalamus or lower brain structures via Uva. Besides monosynaptic projection from Uva to HVC, Uva can also indirectly drive HVC via NIf (Hahnloser et al. 2008). In this chapter, we have shown that NIf is not necessary for mediating the input of timing signal to HVC, because the interhemispheric switching patterns of HVC stimulation effectiveness of song production is not qualitatively different between bilateral pre- and post-lesion of NIf.

Furthermore, in sleeping birds we have shown that HVC-projecting Uva neurons frequently display periods of tonic firing. Such tonic firing is also observed in awake birds and is associated with suppressed bursting of HVC and RA neurons. Our pharmacological and electrical manipulations revealed a suppressive mechanism mediated by tonic spikes mainly in UvaHVC cells. These results suggest that Uva may act as the inhibitory mechanism for the interhemispheric coordination in song production as discussed in Chapter 3. Such an inhibitory function might operate in juvenile birds as a complementary process that is regulated during song development or function in adult birds as real-time interhemispheric coordination for song production.

During juvenile song development, the number of spiking RA-projecting HVC neurons might drift randomly during the song motif with some yet unknown criteria. This dynamic regulation can be achieved by some inhibitory drive that turns off some RA-projecting HVC neurons such that also leads to a compensatory increase in the number of spiking neurons in the contralateral HVC. Since we have shown that Uva activity contributes to state-dependent firing modulation in RA, it might also hav a role involving sensorimotor integration during early vocal learning in a songbird (Gobes and Bolhuis 2008; Shank and Margoliash 2009). In a future experiment, one could unilaterally lesion Uva in a juvenile bird before the onset of song development. After the
bird reaches adulthood, the interhemispheric switching patterning could be examined. If it is diminished, it may unequivocally reveal the role of the avian thalamus in forming the song circuits across both hemispheres.

If Uva has a critical role in interhemispheric coordination in adult birds, then lesioning it should result in a reduction or loss in synchrony of HVC activity across hemispheres, and indeed, bilateral Uva-lesion experiments have reported severe and permanent song impairment (Williams and Vicario 1993; Coleman and Vu 2005). Interestingly, unilateral Uva-lesions on either brain hemisphere only produced immediate and severe song deficits that recovered substantially in about 2 weeks (Coleman and Vu 2005). Previous preliminary findings suggested that unilateral Uva lesions in zebra finches lead to a mismatch in HVC activity patterns between hemispheres during singing attempts (Coleman and Vu 2000). However, it is very interesting that the onset of HVC activity associated with the first syllable of the bird’s distorted song remains synchronized between hemispheres.

This observation raises the possibility that the other pathway (see Figure 4.1D), which bypasses Uva and governs the thalamic nucleus DMP and the AFP nucleus MMAN, may be involved in synchronizing the initial premotor activity in both hemispheres. As described in Chapter 3, HVC or LMAN stimulation can cause interhemispheric complementary stimulation effectiveness, including the song-level effect of early restarts (see Table 3.1 and Figure 3.2D). During HVC stimulation, will bilateral lesions of MMAN impair the brain’s ability to re-initializing the premotor activity? One may answer this question by doing an experiment to check how interhemispheric switching patterns in terms of LE curves differ between pre- and post-lesion of MMAN with HVC stimulation.

If unilateral Uva lesions impair the real-time interhemispheric coordination for song production, how is such coordination restored with song recovery? Previous preliminary findings indicated that MMAN may play a critical role in this recovery (Coleman and Vu 2001). Combining a unilateral Uva lesion and either unilateral or bilateral MMAN lesions did not recover the bird’s song. Also, the recovered song of birds with unilateral Uva lesion was re-impaired by a subsequent unilateral MMAN lesion, with no recovery occurring after the MMAN lesion. Because MMAN is the only other known forebrain song nucleus to project to HVC and to receive bilateral inputs from DMP, MMAN and DMP might also play a significant role in interhemispheric coordination and its plasticity could be especially important for the song recovery from unilateral Uva lesion.

Since bilateral NIf lesions do not severely impair singing (Cardin et al. 2005), and
chronic song-triggered HVC stimulation after bilateral NIf lesions also revealed stimulation effectiveness with interhemispheric switching patterns, it seems that Uva is indeed a bilateral coordinator, and Uva\textsubscript{HVC} cells may be one of the players involved. At least, such an idea is consistent with a putative inhibitory role of Uva\textsubscript{HVC} spikes and with theoretical work that suggests that inhibition has excellent synchronization properties (Van Vreeswijk et al. 1994; Bush and Sejnowski 1996). The other players involved could be the MMAN and DMP cells that may aid in synchronizing the trigger signal to initialize the song-related premotor activity across both hemispheres.

4.4 Methods

All experiments were carried out in accord with protocols approved by the Veterinary Office of the Canton of Zurich, Switzerland, and in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences 1996).

**Subjects.** Zebra finches (Taeniopygia guttata) were obtained from commercial suppliers (Qualipet in Dietlikon; and Animal Diffusion in Villarimboud) and our own breeding colony. Data were taken from a total of 40 adult zebra finches (>90 d).

**Surgery.** Animals were maintained on a day-night reversed 12-h light cycle to assist in obtaining sleep during daytime experimental sessions. Birds were anesthetized with 1 – 3 \% isoflurane in oxygen and small holes (~200 µm) were made in the dura over HVC, RA, and Uva; wound margins were treated with xylocaine gel (2%, Astrazeneca). For Uva experiments, the animal was placed in a small foam restraint and subsequently moved to the recording apparatus without further anesthesia. For paired stimulation-recording experiments across two hemispheres, the animal was placed in a small foam restraint and anesthetized with intramuscular injections of 20\% solution of urethane (5 ml/kg; Sigma, St. Louis, MO; delivered in three injections at 30 min intervals).

**Electrical stimulation and electrophysiology.** For antidromic identification of Uva\textsubscript{HVC} neurons, we inserted bipolar stimulation electrodes into HVC (Teflon-insulated 50 µm diameter stainless steel wires spaced 0.5 mm apart). Electrical stimulation was produced using an isolated stimulation unit (A.M.P.I., Inc), which delivered single monophasic 50–500 µA current pulses of 0.2 ms duration. Identification of Uva\textsubscript{NIf} neurons was performed in different birds using monopolar current pulses of 0.2 ms duration, delivered through 100 kΩ anodal tungsten electrodes placed in the centres of NIf and HVC (cathodal electrodes were placed on the surface of the brain). The same technique was
used to locate the site of NIf, MMAN, Uva, or DM for recording multiunit hash responses evoked by contralateral HVC stimulation. The same bipolar stimulation electrode and the 100 kΩ anodal tungsten recording electrodes were used.

RA and HVC recordings were performed with sharp glass electrodes (5–15 MΩ, borosilicate, 1.0 mm OD, 0.7 mm ID) filled with 3 M KCl. HVC interneurons were identified based on their high spike rates compared to HVC projection neurons (Hahnloser et al. 2006). Uva recordings were either performed with similar glass electrodes or with tungsten metal electrodes (2–5 MΩ, Micro Probe Inc). Extracellular signals were band-pass filtered (0.3–13 kHz) and digitized to 16 bits precision at a sampling rate of 30 kHz on a Pentium-based PC running custom LabVIEW software (National Instruments, Inc.). Electrical Uva stimulation was performed using a monopolar stimulation electrode placed in the centre of Uva. Orthodromic response thresholds in HVC were typically 10 μA.

**Drug Injections.** Injections were made from pulled glass pipettes (roughly 20 μm tip size) using a pressure injection system (Pico Spritzer, Inc.). After Uva recording and injection experiments, a small dose of fluororuby (23 nl) or Alexa Fluor 488 (44 nl) was injected into Uva. In two birds, Alexa Fluor 488 was co-injected with either GABA or glutamate to assess drug leakage. Animals were euthanized by intraperitoneal injection of 20% nembutal or by cranial dislocation. The brain was removed for histological examination of unstained slices to verify the locations of drug injection sites. We verified that in none of the birds injected with glutamate there was leakage of Alexa Fluor into the basal forebrain or the brainstem. To inactivate Uva for prolonged periods, we injected roughly 10 nl of GABA every 10 seconds.

**Statistics.** Unless specified, all statistical tests refer to the Wilcoxon rank sum test of equal medians. Statistical significance was defined by p<0.01. For the analysis of spontaneous activity, we included all cells for which at least 200 s of data with eyes closed were recorded, and for the analysis of auditory responses all cells exposed to at least 16 renditions of the BOS stimulus.

**Instantaneous firing rate (IFR).** In the figures, we represented spike trains by the instantaneous firing rate function, a continuous function defined by the inverse of the interspike interval surrounding time t.
**Burst firing rate (BFR).** A burst spike train is formed by removing single spikes from a spike train (single spikes are spikes that do not form an interspike interval smaller than 10 ms with either the preceding or the following spike). The average firing rate of a burst spike train is referred to as the burst firing rate.

**Interspike interval (ISI) probability density function (pdf).** To display the firing statistics of neurons we computed the ISI pdf $h(\tau)$ ($\tau$ stands for the ISI). Bin centres $\tau_i$ were chosen on a logarithmic scale ($i = 1, \ldots, 100$), $h(\tau)$ is simply a normalized ISI histogram satisfying $\sum_i h(\tau_i)$.

**Cross-covariance function:**

The (cross-) covariance function $C_{AB}(t)$ between two spike trains $\rho_A(t)$ and $\rho_B(t)$ (modelled as a sum of delta functions) is a measure of relative spike density fluctuation.

It is computed as

$$C_{AB}(t) = \frac{1}{T} \int_0^T \rho_B(s + t)\rho_A(s) \, ds - \bar{\rho}_A \bar{\rho}_B,$$

where $\bar{\rho}_A$ and $\bar{\rho}_B$ are the mean firing rate of neurons A and B, and $T$ is the duration of the recording. The first term on the right side of Equation (4.1) is known as the cross-correlation function. We smoothed covariance functions by convolution with a Gaussian window of standard deviation 20 ms in Figures 4.5E, and 4 ms in Figures 4.5C. The smoothed covariance functions were down-sampled by summation of covariance values over 1 ms bins. The significance of (positive or negative) peaks in covariance functions were assessed using 99% confidence thresholds that corresponded to three jack-knife standard deviations. The jack-knife standard deviations were estimated by removing spikes in 20 s windows. Only significant peaks are reported in the text.

**NIf lesion.** A bird with chronically implanted HVC stimulation electrodes was later used in the NIf lesion experiments after enough data for chronic HVC stimulation during singing were collected. NIf was bilaterally mapped before lesion by recording antidromic HVC-stimulation activity. Four to six injections of 50–75 nl of 7 mg/ml ibotenic acid (Biosearch Technologies, Novato, CA) were then made in each NIf by pressure injection through a pressure injection system (Pico Spritzer, Inc.). Each injection was made over the course of 5 min and the injection pipette was slowly withdrawn to minimize spread up the pipette track. Birds recovered in the sound-recording chamber so that all post-lesion vocalizations with HVC stimulation were recorded.
**PSTH analysis.** Poststimulus time histograms (PSTHs) for stimulation-evoked activity were constructed by aligning with the stimulation onset. A raster was computed using a simple thresholding algorithm that identified neural events (spikes or peaks) that occurred within an absolute threshold range. PSTHs were generated from the activity collected from a total of 50 stimuli (delivered at 1 Hz), which was then compiled into 1ms bins. To test significance of the stimulation response, we calculated post-to-baseline ratios by taking the maximum value of the first 100 bins (100 ms) after stimulation and dividing it by the mean of all bins in the 50 ms preceding stimulation. As a control, we thus calculated a control-to-baseline ratio using this same baseline period and dividing its mean by the maximum value within this baseline window. The paired Student’s t-test was used to test the significance between the post-to-baseline and control-to-baseline ratio. Values in the text are stated as the mean ± SD. Response latency for a given set of stimuli was determined by firstly summing the rectified neural traces after all stimuli and smoothing with a 1 ms wide Gaussian filter and thus computing the time from the beginning of stimulation to reach the half value of the peak of this smoothed trace.
Chapter 5: Interhemispheric switching for birdsong production is uncorrelated with lateralized auditory feedback

“We live in a noisy environment, with too much information, and the main task is to pull information out of that. The brain does that by making maps that accentuate the useful information. Once you do that, it makes sense to store it.”

John Allman (1942-)

Chapter 5 : Interhemispheric switching for birdsong production is uncorrelated with lateralized auditory feedback

Songbirds such as zebra finches need auditory feedback to imitate tutor song and maintain the learned song in adulthood. Auditory feedback provides a means to compare motor intent (memory of tutor song) with actual motor performance. Throughout the bird’s entire life, the neural activity responsible for song production requires precise interhemispheric coordination possibly involving in the process of auditory feedback. Hence, the bilateral neural representations of tutor-song memory to which the auditory feedback is compared might be relevant to interhemispheric coordination for birdsong production. Based on the hypothesis that deprived sensory input from one side of the brain may result in a biased brain dominance distributed across hemispheres (Neff and Casseday 1977; Frenkel and Bear 2004; Vale et al. 2004), we hope to see that the interhemispheric switching stimulation effectiveness during vocal production in a unilaterally or bilaterally deafened zebra finch can be diminished. We performed HVC stimulation in birds either bilaterally and unilaterally deafened after 120 dph, or unilaterally deafened before 20 dph (before the onset of sensory phase), none of these cases lacked the complementary stimulation effectiveness for song production. These experiments could unequivocally rule out the possibility of “auditory feedback” as a cause of interhemispheric switching for song production.

5.1 Introduction

Previous work done by Vu’s group has shown that unilaterally stimulating HVC in singing birds distorts the acoustic structure of ongoing syllables, suspends ongoing motifs, and rearranges the temporal pattern of ongoing song bouts (Vu et al. 1994). They also provided evidence that such changes in the temporal pattern of the song bout following a stimulus to HVC was not due to auditory feedback, because stimulating HVC still altered
song patterning even in deafened birds. However, up to the time Vu published these results, interhemispheric switching stimulation effectiveness alternating between HVC in two hemispheres (Wang et al. 2008) had not yet been discovered. Although electrical perturbation in HVC resulted in changes in song patterning in deafened birds, we have no ideas whether auditory ablation can influence the interhemispheric switching patterning reported in Chapter 3 of this thesis.

During song development, auditory feedback provides a means for juvenile birds to compare motor intent (memory of tutor song) with actual motor performance. In adult birds, hemispheric differences in avian song discrimination tasks have been reported (Cynx et al. 1992), but whether there is hemispheric dependence for the memory formation of tutor song is yet unknown. One piece of evidence for lateralized memory formation is that in chicks, the main sequence of memory stages depends on the elaboration of the memory in the left hemisphere (Gibbs et al. 2003).

The development of auditory memory, distributed in the ascending and descending neural pathways, is complex. A previous study indicated that stimulus-guided asymmetry is present as early as at the level of the cochlea before it is evident in the auditory cortex. Unilateral cochlear ablation can produce loss of inhibitory synaptic conductance in the contralateral inferior colliculus in the midbrain (Vale et al. 2004). Furthermore, effects of unilateral ablation on auditory cortex on monaural cat’s ability to localize sound suggested that the auditory cortex contralateral to a given ear is necessary for the animal to recognize that a stimulus is presented to that ear (Neff and Casseday 1977). For adult zebra finches, auditory feedback is required to maintain their learned songs (Leonardo and Konishi 1999; Tumer and Brainard 2007; Sober and Brainard 2009). Interhemispheric coordination may be involved in the processing of bilateral auditory feedback, where hemispheric differences may reside.

In order to investigate whether lateralized auditory feedback is involved in interhemispheric switching for song production, we stimulated HVC in adult birds (>120 dph) that were either unilaterally deafened before 20 dph (during the time before sensory acquisition of tutor songs begins) or bilaterally deafened after 120 dph. If the interhemispheric switching patterns were diminished by the removal of bilaterally coordinated auditory feedback, regardless of whether the birds were bilaterally deafened in adulthood or unilaterally deafened before the onset of their sensory phase, we would be able to identify a causal link between the asymmetric auditory processing and the hemispheric specialization for birdsong production.
5.2 Results

We examined the effect of HVC stimulation in adult birds receiving either bilateral (n=1) or unilateral (right-side, n=1) deafening at the age of 120 dph. As shown in Figure 5.1, both birds still exhibited interhemispheric switching of stimulation effectiveness. The correlation coefficients between left and right LE curves are -0.18 and -0.56. The amplitudes of stimulation current were reasonably low in the range between 200 and 250 μA. Therefore such interhemispheric switching was not due to real-time auditory detection of a mismatch between motor intent and motor performance that led to feedback correction of the motor program for vocal production.

Because the motor intent and basic motor performance for these adult birds were created before deafening, the switching dynamics may already have been hardwired during song development (before deafening), after which it could withstand any auditory ablation. Therefore we examined the HVC stimulation effectiveness from another two birds undergoing unilateral (right side) deafening at the age of 15 and 20 days, which is before or just at the onset of the sensory phase. We hypothesized that unilateral auditory ablation before the birds start the acquisition of tutor songs may cause a hemisphere-biased motor intent (memory of tutor songs) for the following song development. Such a bias across hemispheres may force the hemispheric dominance for vocal practice restricted to only one hemisphere and thus eliminates the necessity of interhemispheric switching.

In Figure 5.2, we can see that an interhemispheric switching effect still exists in these two birds which were unilaterally deafened before or just on the onset of sensory phase. The correlation coefficients between left and right LE curves were -0.34 and -0.43. The amplitudes of stimulation current were even as low as 100 and 150 μA. These results demonstrate that interhemispheric switching dynamics may be a process that is perhaps irrelevant or very robust to the auditory intervention.
Chapter 5: Interhemispheric switching for birdsong production is uncorrelated with lateralized auditory feedback

Figure 5.1. Interhemispheric switching stimulation effectiveness from adult birds either bilaterally or unilaterally deafened on 120 dph.

(A) For this adult bird having bilateral deafening, the interhemispheric switching stimulation effectiveness still persisted with CC value -0.18 between left and right LE curves. Stimulation pulse is a single biphasic pulse (0.2 ms/phase, 250 μA). (B) Another adult bird receiving unilateral deafening (right side) also showed salient interhemispheric switching stimulation effectiveness with CC value -0.56. Stimulation pulse is a single biphasic pulse (0.2 ms/phase, 200 μA).
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Figure 5.2. Interhemispheric switching stimulation effectiveness from two adult birds unilaterally deafened before sensory phase.

(A) For this adult bird undergoing right-side deafening before the sensory phase (on 20 dph), interhemispheric switching still persisted with CC value -0.34 between left and right LE curves. Stimulation pulse is a single biphasic pulse (0.2 ms/phase, 100 µA). (B) Another adult bird receiving right-side deafening (on 15 dph) also showed salient interhemispheric switching with CC value -0.43. Stimulation pulse is a single biphasic pulse (0.2 ms/phase, 150 µA).
In Chapter 3, we reported the hemisphere-specialized coherence between HVC stimulation and specific sound features. It has also been reported that sound signals are perceived with better temporal resolution in left auditory cortex and with better spectral resolution in right auditory cortex (Zatorre et al. 2002). However in this study, we cannot further examine whether such lateralization for sound features is influenced in birds with early unilateral auditory ablation due to experimental and statistical limitations. To this end, it would be worthwhile to reexamine a larger body of interhemispheric stimulation data from more birds receiving unilateral deafening at the early sensory phase.

5.3 Discussion

The findings in this chapter showed that interhemispheric switching patterning for song production persists both in bilaterally deafened adult birds and in unilaterally deafened birds with right-side cochlear ablation at the age before sensory phase (~15-20 dph). A long-standing issue in the motor control of birdsong is how bilaterally organized behavior attributes to interhemispheric coordination. The results in this chapter suggest that the interhemispheric switching dynamics during singing only requires “non-auditory feedback” pathways for serving the interhemispheric coordination during singing.

It has been known that multiunit activity in left and right HVC are strongly synchronized during singing (Schmidt 2003) and perturbing neural activity in one HVC will cause a rapid ‘reset’ of neural activity in the contralateral HVC (Vu et al. 1998). Hence, activity in the two HVCs should be monitored and rapidly brought into register when in conflict. For example, bilaterally organized mid- and hindbrain areas could detect specific premotor patterns and trigger a particular motor event in the contralateral hemisphere via the relay station Uva in thalamus to coordinate the entire song production process (see Chapter 4). These feedback pathways are not auditory because HVC stimulation also reveals persistent interhemispheric switching patterns in deafened birds no matter whether deafening was done unilaterally before sensory phase or bilaterally done in adulthood.

If auditory input has little or no relevance for the formation of interhemispheric switching dynamics, it would be interesting to see whether sibling birds from the same brood, which receive identical auditory input from a single tutor, would develop different switching patterns for the same motor intent (see Chapter 6). Based on the same (or highly similar) motor intent of learned vocal output, if we could observe qualitatively similar interhemispheric switching patterns, it would be less complex for us to uncover the driving source of interhemispheric switching dynamics.
5.4 Methods

All experimental procedures for animal operations and methods for HVC stimulation data analysis have been described in section 3.3.

Surgery for cochlear removal:

Two male birds were either bilaterally or unilaterally (right side) deafened by cochlear removal from both inner ears in adulthood (>120 dph). Because we cannot visually identify the sex of zebra finches at the age of 20 dph, 10 juvenile birds at ages between 15 and 20 dph were selected to be unilaterally deafened by cochlear removal from their right inner ears. Two male birds were finally identified and used for the experiments. Their right cochleae were removed on 15 and 20 dph, respectively.

We developed our own technique for cochlear removal, which was different from the classical methods as described in (Konishi 1965). Under anesthesia with 1 – 3 % isoflurane in oxygen, the pocket-like skin of the outer ear is pulled and cut away with extra fine scissors (Item No. 14084-08, Fine Science Tools Inc., USA). A hole was made in the tympanic membrane, and the columella was extracted with a fine forceps. A hooked tungsten wire was then inserted through the oval window, and the cochlea was pulled out. After the cochlea was removed, the wound skin of the outer ear was immediately closed with NEXABAND S/C topical tissue adhesive (Abbott Laboratories, North Chicago, IL, USA). After surgery, juvenile birds were put back to their rearing cage and reared by their parents until they were older than 65 dph.
Chapter 6: Idiosyncratic interhemispheric switching dynamics for birdsong production

It is often assumed that learning process takes place by modifying a preceding stable neural representation of familiar behaviors. However, other studies have suggested that motor learning functions by taking advantage of the potential for change in unstable neural representations (Padoa-Schioppa et al. 2004; Rokni et al. 2007). The justification given was that equivalent behaviors can originate from different neural representations. Instead, we have studied the stability of neural dynamics in birds. We asked ourselves: How different are the neural dynamics for a motor learning behavior across individual animals? Here, we report that the interhemispheric switching dynamics revealed by electrical stimulation in HVC during vocal production are stable over time. We found that the dynamics in sibling birds singing temporally and spectrally similar songs were intrinsically different. Furthermore, identical syllables within a song motif produced by individual birds can also be governed by either different or replicated interhemispheric dynamics. We propose that such variation of neural dynamics may be the result of stable but idiosyncratic neural representations for development across two cerebral hemispheres.

6.1 Introduction

Neural recordings in behaving animals have revealed much about the mechanisms underlying motor learning. Changes in neural activities have been correlated with learning sensorimotor associations (Paz and Vaadia 2004) and learning movement sequences and skills (Nakamura et al. 1998). The assumption implicit in these studies is that there is an underlying stable neural representation for familiar or specialized behaviors and changes in the neural representation necessarily reflect motor learning. Hemispheric specialization for human speech, for example, is one of the most intriguing aspects of stable brain lateralization. In the mid nineteen century, the French physician Paul Broca discovered a region on the left side of brain that is essential for language (Broca 1861); damage to Broca’s area severely leaves people unable to talk, but the same
region on the right side is not so vital. In recent years, further evidences from diverse methodologies have revealed that left and right auditory regions may be individually specialized for processing of sounds based on acoustic properties. Rapidly changing signals are processed preferentially in auditory areas of the left hemisphere, and tonal stimuli are best processed in auditory areas of the right, reflecting a highly fixed hemispheric specialization (Zatorre et al. 2002; Sininger and Cone-Wesson 2004). Such lateralized specialization is stable over time if the brain does not suffer from injury that some neural plasticity may take place to compensate the function damage.

However there are several indications that neural representations may, under some circumstances, be unstable even without obvious learning. For example, previous studies showed that when monkeys performed a familiar reaching task, the directional tuning of neurons in the supplementary motor area changed substantially (Padoa-Schioppa et al. 2004). These results suggest that that motor learning is based on a surprisingly unstable neural representation and that during a familiar task, tuning curves exhibited slow random drift, whereas during learning of a novel task, random drift was accompanied by systematic learning-related changed (Rokni et al. 2007).

Speech development in human and song development in birds are some of the most sophisticated motor learning tasks that have kept neuroscientists busy for decades. In birdsong research, the issue about long-term stability of synaptic configurations formed during song development has not been addressed yet. In terms of interhemispheric switching dynamics reported in previous chapters, here we will firstly examine whether interhemispheric switching patterns revealed by HVC stimulation are stable over time. Then we will study the variation in interhemispheric switching dynamics revealed for song production across individual birds. This can be approached on two different levels: on the song level, we will investigate whether sibling birds with highly similar singing outputs also develop close interhemispheric switching dynamics. On the syllable level, we will analyze that when a bird produces identical syllables in a motif, whether the dynamics of each syllable is simply replicated or not. Our results can make it clear whether the learning behavior of a neural system is governed by deterministic or stochastic processes.

6.2 Results

Before investigating the animal-to-animal variation of neural dynamics like the interhemispheric switching patterning underlying song production, we have to examine whether it is stable over time. From our stimulation data pooled for 23 birds, we have
confirmed that the interhemispheric switching patterns can be stable at least over one to two weeks.

In this section, we examine a bird that underwent HVC stimulation for a month. Its interhemispheric switching patterns did not significantly change over that period. As shown in Figure 6.1, the similarity (CC value) between LE curves (red vs. blue solid curves) for left and right hemispheres is 0.45 and 0.73, respectively. This implies that the locations of LE peaks change little over time.

The amplitude of stimulation currents required to elicit similar stimulation effectiveness was 550 μA, which is higher than the stimulation amplitude at the beginning of only 150 μA. This was because the chronically implanted stimulation electrodes eventually become surrounded by growth of cellular debris or glia cells, and thus the current-distance constant increased (Ranck 1975; Tehovnik 1996) such that small stimulation currents were insufficient to interrupt the song.

In fact, the first time when we tuned the stimulation amplitude to 500 μA, the stimulation effectiveness obtained from that day was much weaker than those from one month ago, especially for the right hemisphere (see the dashed LE curves in Figure 6.1). We thus carefully performed electrolysis to refresh the stimulation electrodes by injecting current of amplitude 10~20 μA for approximate 5~10 seconds and repeated it for 5~10 times at interval of 5 minutes. Surprisingly, not only did the bird still sing normally, but also the next day we could again obtain complete interhemispheric stimulation effectiveness illustrated in Figure 6.1 by red solid curves which are very similar to those in blue as the one-month-ago case. These results suggest that the interhemispheric switching dynamics revealed by the stimulation effect should be a robust neural mechanism.
Figure 6.1. Interhemispheric switching is stable over time.

The stack plot of sound amplitude together with the blue LE curves are computed from the HVC stimulation data obtained during 10.09.2009–11.09.2009. The CC value of this case is -0.42 that implies interhemispheric switching dynamics. The dashed LE curves represent the stimulation effectiveness from the data obtained during 05.10.2009–06.10.2009. Its weaker effectiveness was due to the degradation of stimulation electrodes (see text). The red LE curves represent the stimulation effectiveness from the data obtained during 08.10.2009–10.10.2009 with stimulation electrodes refreshed on 07.09.2009. The CC value between blue and red LE curves for left and right hemispheres are 0.45 and 0.73, respectively, implying that interhemispheric switching dynamics is stable over time.
Considering that the interhemispheric switching dynamics are stable, does vocal imitation follow a preset program that develops deterministic interhemispheric switching dynamics corresponding to a given target song? Our results of HVC stimulation from the sibling birds raised in the same clutch as well as the lineal birds all showed that the interhemispheric switching patterns can be very different across similar songs. “Similar song” refers to a song similarity (see Methods) greater than 75%, which was used as an empirical standard in a previous study (Liu et al. 2004).

As shown in Figure 6.2A - 6.2C, two sibling birds developed very similar song (similarity=88.45±4.89%), and their HVC stimulation effectiveness in terms of LE curves showed clear interhemispheric switching patterning. In Figure 6.2D, for both hemispheres, the CC between LE curves corresponding to two birds is negative, implying that these birds developed their songs with interhemispheric switching patterning of different temporal rhythms, despite the highly similar acoustic structures.

In Figure 6.3, we show an example from another pair of sibling birds which learned an identical sound segment (similarity=95.05±1.81%) about 270 ms long, but the corresponding interhemispheric switching patterns (LE curves) were also different. The CCs between LE curves corresponding to the aligned sound segments for a given hemisphere in different birds are -0.46 (HVC left) and -0.23 (HVC right).

Figure 6.4 shows another case from lineal birds (uncle and nephew) that both learned a similar sound segment (similarity=83.82±4.08%) about 400 ms long, but the corresponding interhemispheric switching patterns (LE curves) were also different. The CCs between LE curves corresponding to the aligned sound segments for right hemisphere in each bird are -0.15 (HVC long) and -0.07 (HVC short). Notice that in this case although we only compare LE curves for right hemisphere in two birds, we still obtained two comparisons, because one bird had implantation of two stimulation electrodes in right HVC, one with long inter-electrode distance (HVC long) and the other with short inter-electrode distance (HVC short). These results all showed that on the entire motif or syllable level across individual birds, highly similar sounds in length ranging from 0.2~0.7 s can be governed by very different interhemispheric switching dynamics revealed by the patterning of HVC stimulation effectiveness.

Across individual animals, although in our study we used birds with relationship of consanguinity, there still exists large animal-to-animal variability in the neural circuit parameters (Goaillard et al. 2009). Therefore we further analyzed two birds (the same birds used in Figure 6.4) whose song motifs contained repetition of identical syllables to check whether each bird would also develop different interhemispheric switching strategies for each syllable repetition.
As shown in Figure 6.5, the first bird revealed the tendency to produce identical syllable sets (similarity=94.39%±4.32%) with same interhemispheric switching patterns. For left HVC stimulation, although the CC between LE curves corresponding to the identical syllable sets were not well correlated (CC=-0.03), the salient LE peaks were temporally matched, as indicated by black arrows in Figure 6.5B. The right HVC stimulation effectiveness showed salient positive correlation (CC=0.44) between LE curves corresponding to the identical syllable sets.

The second bird used different interhemispheric switching strategies for producing identical syllable sets (similarity=94.39%±4.32%) which shared similar acoustic structures with those in the first bird (similarity=83.82%±4.08%; see also Figure 6.4). For the second bird, the LE curves corresponding to each identical syllable set from HVC stimulation using ‘long’ or ‘short’ electrode (see also the description in the previous paragraph) in the right hemisphere were highly anticorrelated (CC=-0.61 and -0.48) as shown in Figure 6.6. Such anticorrelation implies out-of-phase temporal patterns of stimulation effectiveness for the same vocal outputs.

In summary, by analyzing the correlation between the LE curves corresponding to highly similar vocal outputs from either different or same birds, we explored the different mapping between interhemispheric switching patterning and song that suggests birds may follow idiosyncratic developmental trajectories for vocal imitation.
Figure 6.2. Sibling birds use different interhemispheric switching dynamics for similar vocal outputs (Example 1).

(A) and (B) show sound amplitude stack plots and corresponding LE curves for HVC Stimulation in zebra finch siblings o3r3 (blue) and o1r3 (red), respectively. They were members of the same clutch and mastered highly similar copies of their father’s song. The similarity between their songs is 88.45%±4.89% (see Methods). The CC between left and right LE curves in o3r3 and o1r3 is -0.33 and -0.40, respectively that both show obvious interhemispheric switching patterning for song production. (C) Top panel: an example of original song spectrogram for o3r3; bottom panel: an example of original song spectrogram for o1r3; middle panel: aligned version (see Methods) of song spectrogram for o1r3 to best match the spectrogram in top panel. The red rectangle marked the range for which we compare the corresponding LE curves as shown in (D). (D) To compare whether these birds used same interhemispheric switching patterning for similar song, LE curves were aligned in the same way as the spectrograms shown in (B). Top panel: blue LE curve for left HVC stimulation in o3r3 is anticorrelated with the red LE curve for left HVC stimulation.
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in o1r3 (CC=−0.10). Bottom panel: blue LE curve for right HVC stimulation in o3r3 is anticorrelated with the red LE curve for right HVC stimulation in o1r3 (CC=−0.16). Both negative CCs reveal that in either left or right hemisphere, these two sibling birds developed different interhemispheric switching patterning for song production.

Figure 6.3. Sibling birds use different interhemispheric switching dynamics for similar vocal outputs (Example II).

(A) and (B) show sound amplitude stack plots and corresponding LE curves for HVC stimulation in zebra finch siblings y14k-s17 (blue) and y12k-s17 (red), respectively. They were members of the same clutch and developed songs sharing a highly similar sound segment about 270 ms long. The similarity between these two sound segments is as high as 95.05%±1.81%. The CC between left and right LE curves in y14k-s17 and y12k-s17 is -0.62 and -0.36, respectively that both show obvious interhemispheric switching patterning for song production. (C) Top panel: an example of original song spectrogram for y14k-s17; bottom panel: an example of original song spectrogram for y12k-s17; middle panel: aligned version of song spectrogram for y12k-s17 to best match the spectrogram within the red rectangle in top panel. The red rectangle marked the aligned sound segment for which we compare the corresponding LE curves as shown in (D). (D) To compare whether these birds used same interhemispheric switching patterning for similar song segments, LE curves were aligned in the same way as the spectrograms shown in (B). Top panel: blue LE curve for left
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HVC stimulation in y14k-s17 is anticorrelated with the red LE curve for left HVC stimulation in y12k-s17 (CC=-0.46). Bottom panel: blue LE curve for right HVC stimulation in y14k-s17 is anticorrelated with the red LE curve for right HVC stimulation in y12k-s17 (CC=-0.23). Both negative CCs reveal that in either left or right hemisphere, these two sibling birds developed different interhemispheric switching patterning for song segments with almost identical acoustic structures.

Figure 6.4. Lineal birds use different interhemispheric switching dynamics for similar vocal outputs. (A) and (B) show sound amplitude stack plots and corresponding LE curves for HVC stimulation in zebra finch lineal relative yellow18 (blue) and o8k-y6 (red), respectively. Bird yellow18 is o8k-y6’s uncle. The birds developed songs sharing a highly similar sound segment in about 400 ms long. The similarity between these two sound segments is 83.82%±4.08%. The CC between left (black) and right (red) LE curves in o8k-y6 is -0.46 that shows obvious interhemispheric switching patterning for song production. For yellow18, we only performed right HVC stimulation at two locations in HVC that one was by a bipolar stimulation electrode with longer inter-electrode distance than the other. The CC between these two cases is 0.37 (see Chapter 3). (C) Top panel: an example of original song spectrogram for yellow18; bottom panel: an example of original song spectrogram for o8k-y6; middle panel: aligned version of song spectrogram for o8k-y6 to best match the spectrogram within the red rectangle in top panel. The red rectangle marks the aligned sound segment in which we compare the corresponding LE curves as shown in (D). (D) Top panel:
blue LE curve for right HVC stimulation (long inter-electrode distance) in yellow18 is weakly anticorrelated with the red LE curve for right HVC stimulation in o8k-y6 (CC=-0.15). Bottom panel: blue LE curve for right HVC stimulation (short inter-electrode distance) in yellow18 is uncorrelated with the red LE curve for right HVC stimulation in o8k-y6 (CC=-0.07). Both negative CCs reveal that in either the left or right hemisphere, these two lineal birds developed different interhemispheric switching patterns for song segments with highly similar acoustic structures.

Figure 6.5. Bird ‘o8k-y6’ uses similar interhemispheric switching dynamics for identical syllable sets within one song motif.

This bird is also used for the result shown in Figure 6.4B. (A) Top panel: an example spectrogram of its song motif shows a successive repetition of a syllable set comprising three notes with one note overlapped.
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The blue rectangle marked the syllable set while the red rectangle marked the successive repetition of it. Middle panel and bottom panel are those zoomed out spectrograms from those in the blue and red rectangle, respectively to visually demonstrate that these two syllable sets should be identical (similarity=94.39%±4.32%). These two sound segments are aligned and selected by the methods described in Methods. (B) Top panel: Two aligned LE curves from left HVC stimulation, of which the colors correspond to the color-zoomed sound segments in (A) are not well correlated with a small CC=-0.03. Bottom panel: right HVC stimulation, the two LE curves are highly correlated with CC=0.44.

Figure 6.6. Bird ‘yellow18’ uses different interhemispheric switching dynamics for identical syllable sets within one song motif.

This bird is also used for the result shown in Figure 6.4A. (A) Top panel: an example spectrogram of its song motif shows a successive repetition of a syllable set comprising three notes. The blue rectangle
marked part of the syllable set while the red rectangle marked the successive repetition of it. Middle panel and bottom panel are those zoomed out spectrograms from those in the blue and red rectangle, respectively to visually demonstrate that these two syllable sets should be identical (similarity=97.08%±1.26%). These two sound segments are aligned and selected by the methods described in Methods. 

(B) Top panel: Two aligned LE curves from right HVC stimulation with stimulation electrode of long inter-electrode distance; these LE curves of which the colors corresponding to the color-zoomed sound segments in (A) are highly anticorrelated with CC=-0.61. Bottom panel: the case from right HVC stimulation with stimulation electrode of short inter-electrode distance; these LE values are also anticorrelated with CC=-0.48.

**6.3 Discussion**

In this chapter we demonstrated that the interhemispheric switching dynamics are stable over time, in the sense that the same switching patterning is reproducible over a month. In contrast to primate motor learning systems that are based on a surprisingly unstable neural representation (Rokni et al. 2007), the stable interhemispheric switching dynamics in zebra finches may be due to the fact that once they have learned a song, they will not be able to learn new songs anymore.

The sibling birds which produced highly similar songs developed intrinsically different interhemispheric switching patterning in song production. This result first provides neural evidence that clarifies a previous report that in a family clutch, a young zebra finch chooses a strategy different from that of his siblings, perhaps in order to better track his own vocal development as he learns the song (Liu et al. 2004). This is actually a very biologically plausible reason to explain why infants can show remarkable variability, unrelated to the parent’s efforts to guide speech development, in learning the sounds of language (Menn and Stoel-Gammon 2001).

Moreover, on the syllable level, individual birds which produced two identical syllables in a song motif also developed different strategies to implement identical vocal output. We showed that one bird used the same interhemispheric switching pattern in the repetition of two identical sound segments within one song motif, whereas another bird (this bird’s uncle) used two different temporal switching patterns to implement the two identical sound segments.

Here, it is very important to compare our results with a previous study about the correlation between RA ensemble coding and syllable structures (Leonardo and Fee 2005). Their results proposed that unless two syllables are exactly identical, different sounds of the same type (like harmonic stacks with different fundamental frequencies)
are not generated by correlated pattern of neural activity. Instead, zebra finches learn to activate a different RA neural ensemble at each time point in the bird’s song, independent of the acoustic structure that is being produced. Essentially, the authors claimed that similar sounds are encoded by entirely different patterns of neural activity unless the sounds are identical. In terms of interhemispheric switching dynamics, our results confirm the part that “similar” sounds are subserved by different interhemispheric switching patterning, but even for identical song structures, degenerate mapping may still exist between interhemispheric switching patterns and vocal output. (The term “degenerate” here is used in the same sense that two or more different physical states can be at the same energy level.)

So what might be the behavioural mechanism to drive a brain to use different dynamical programs for similar vocal output? Our results showed that the ontogeny of vocal imitation in zebra finches is more like a problem solving process with many possible solutions. Earlier studies have suggested that song imitation in zebra finches might occur at least two different ways: one method involves many repetitions of an early syllable, and the other method provides a coarse approximation of the whole motif. Additionally, another method may involve switching back and forth between the two methods (Tchernichovski et al. 2001; Liu et al. 2004).

We speculate that the bird which replicates the identical interhemispheric switching pattern for the two identical sound segments (e.g. identical set of syllables) may begin the imitation process with the first method, by successively repeating an identical precursor syllable and then gradually refining the detailed sound structures. Therefore, the temporal pattern of interhemispheric switching is similar for these originally repetitive syllables. However, the bird using the second method to imitate these same syllables would start with two different sets of coarse approximations to these syllable sets and then independently refined each set. It may thus result in interhemispheric switching patterns that are independent among identical syllables. Figure 6.7 illustrates these ideas in a diagram with some putative song learning trajectories. Towards the end of song learning process, the patterns of emerged interhemispheric switching may remain unchanged throughout the rest of the learning process, but only the neural networks located in each hemisphere will still evolve to improve acoustic structures of the song.

In Chapter 3, we have presented evidence that individual hemispheres may be specialized for certain sound features such as AM, FM, and Wiener entropy. However, how can it be possible that identical sounds can be subserved by different patterns of switching dominance? Maybe it is because there is no simple linear relation between acoustic structure in the song and the control parameters used by the bird to produce the
structure. For example, the bird’s syrinx is known to be highly nonlinear in its response to linear control signals (Fee et al. 1998). Furthermore, the premotor neurons (such as HVC and RA neurons) are likely to represent these control parameters in more abstract motor coordinates, rather than direct auditory ones, to innervate the downstream motor neurons, and therefore a nonlinear degenerate mapping could exist.

**Figure 6.7. Variation of learning trajectories can lead to the same end.**

The tutor song contains two identical syllables (BB), each of which comprises three notes B1, B2, and B3. The juvenile birds imitating the successive repetition of this syllable can possibly use two distinct strategies. The first bird (red) can follow a “serial repetition” trajectory by initially repeating an approximate version (β) to syllable B. At this early stage, the interhemispheric switching pattern may also repeat as a unit.
corresponding to \( \beta \). However, the second bird (dark blue) can follow the other “early on a motif” trajectory by initially improvising two different approximations (\( \beta_1 \) and \( \beta_2 \) ) to syllables BB. Therefore, the interhemispheric switching pattern may also comprise different sub patterns corresponding to \( \beta_1 \) and \( \beta_2 \). Towards the end of the song development, the interhemispheric switching pattern represents the registered usage of two hemispheres.

These results may also support the hypothesis that vocal learning does not unfold in a deterministic manner but rather emerges from many stochastic elements that minimize vocal error to reach a global minimum where the vocal output matches the template. This suggests that when birds are trying to accomplish a motor learning task requiring replication of the tutor song, their overall premotor synaptic configurations can form via very different pathways across cerebral hemispheres during the learning process. During song development, the neural circuitry is selected out of several redundant cross-hemisphere networks of different configurations and synaptic strengths. Therefore, the cross-hemisphere synaptic configurations underlying song learning can be stochastically modified that eventually causes cruising among the synaptic configuration space with equivalent behaviors but different neural representations. The outcomes of these assumptions will just reflect an idiosyncratic nature of birdsong which has also been proposed in the sensory domain (Margoliash 1986).

A simple model for a similar case observed from primate motor learning has been proposed to explore the implications of these assumptions (Rokni et al. 2007). Additionally, in the birdsong model, we can also explore how brain circuits optimally implement such stochastic learning by analyzing detailed song development steps influenced by systematic circuit perturbation such as our song-triggered stimulations. So why does biology do it this way? Because genetically identical cells or animals show substantial variability in many underlying cellular parameters, and variable solutions to the production of similar phenotypes is probably an important substrate for evolutionary selection (Greenspan 2001; Chouard 2008).

### 6.4 Methods

All experimental procedures for animal operations and methods for HVC stimulation data analysis have been described in section 3.3.

**Similarity measurement.** All the songs used in this study are considered to be “directed” songs which supposedly have maximum stereotypy, since we allow all birds to have
visual contact with one or more female zebra finches through the glass door of the experimental chamber. Similarity for a pair of song motifs or sound segments was quantified using the latest update of Sound Analysis Pro 2A.01 software with default parameter settings (Tchernichovski et al. 2000). This procedure can measure the similarity between two songs on the basis of pitch, frequency modulation, amplitude modulation, Wiener entropy, and goodness of pitch. The similarity measure between two songs estimates the percentage (%) of sound in one song for which there is a close correspondence in the other. It is calculated in 10 ms intervals and averaged over the duration of the entire song. Sounds are aligned in time so as to maximize the similarity, allowing for 5% time warping. For each type of song comparison (e.g. song similarity between sibling birds or between two sound segments from one bird), we randomly select 100 pairs of desired songs or sound segments to compute the similarity and its value in the text is stated as the mean ± SD.

Alignment and similarity between interhemispheric switching patterns for two similar songs. To compare the correlation between the interhemispheric switching patterns (LE curves) for two similar songs, we have to first define a method to align these two songs of which the LE curves will also be align in the same way. In general, we compute the song energy by summing over all power of frequencies at each time point in the spectrogram. For song alignment, we manually selected a time point during which the energy patterns in the vicinity between two songs are similar but saliently different from those outside the selected time range. The time points of maximum energy value of selected energy patterns are thus aligned. Then we used linear time warping by using the Matlab function resample.m to stretch one song in order to maximally match the other one on the basis of their spectrogram. The goodness of matching $M$ between a given time-warped spectrogram $S_w(f,t)$ and the other matched spectrogram $S_m(f,t)$ is defined as follows:

$$M = \frac{\sum_f \sum_t (S_w(f,t) \cdot S_m(f,t))}{\sqrt{\sum_f \sum_t S_w(f,t)^2} \cdot \sqrt{\sum_f \sum_t S_m(f,t)^2}}.$$

The LE curves of two corresponding songs were also aligned with this method to compute the similarity (correlation coefficient) between LE curves (see details in section 3.4).
Chapter 7: Determination of the unitary elements of birdsong

Much of our behavioral repertoire is made up of sequences of individual motor elements. Birdsong comprises temporally ordered information in well-defined song circuits. Songbirds have provided a good animal model to understand how complex motor sequences are learned and produced. However little is known about the basic unitary elements of birdsong sequentially controlled by a bird’s motor program. In a previous study, Cynx reported that ongoing zebra finch song can be interrupted by a burst of stroboscopic light and that the interruptions occur at discrete locations in the song (Cynx 1990). The locations almost always fell into the silence interval between acoustically continuous syllables, suggesting that syllables may be unitary elements of birdsong. In contrast to Cynx’s stimulation paradigm (acute visual stimuli), we interrupted birdsong by turning the light off for a tenth of a second. In contrast to the findings of Cynx, we found that song syllables were not necessarily the unitary elements being interrupted. Song interruptions occurred in the midst of the syllables, but certain parts of the song motifs were resistant to the perturbation. Most perturbation effects were qualitatively similar to the acute electrical stimulation effects described in Chapter 3. We also found that for several birds subjected to electrical stimulation in HVC, the stimulation-induced syllable transitions could be observed in catch trials, although much more rarely. Thus, HVC stimulation seems to selectively increase the likelihood of occurrence of some rare syllable transitions encoded by the synaptic chain networks of HVC projection neurons (Jin 2009). The effective chain length can be estimated as a length of contiguous set of high effective stimulation times. We found that the effective chain-length distribution followed a scale-free size distribution with scaling exponent around \(-1\) that suggests the chain networks were organized by heterosynaptic competition (Fiete et al. 2010). Our results suggest that song may be represented in the motor program that controls probabilistic transitions of basic song elements encoded in a repertoire of neural networks. The formation of these
networks might result from idiosyncratic self-organizing song learning processes for individual birds (see also Chapter 6).

7.1 Introduction

Sequential behavior is essential to intelligence, and it is a fundamental part of human activities ranging from everyday skills like playing an instrument, swing a golf club, and speaking a language, to complex problem solving like reasoning. Birdsong comprises temporally sequential information and therefore vocal learning in songbirds is a valuable model for understanding how humans can learn sequences of behavior. Determining the elements or primitives of birdsong will provide insight into the essential parts of the neural functional code that constitutes the birdsong system. The results will also reveal the constraints of the neurobiological mechanisms of birdsong. Zebra finches produce highly stereotyped songs that consist of syllables separated by short silence intervals. A syllable is defined as a morphologically discrete trace on a sound spectrogram that lasts 50-120 milliseconds. Such elementary sound structures have only been determined via sound spectrograms, and their functional hierarchies in the neural process remain largely unknown.

Zebra finches and other songbirds in the wild or in human-reared conditions almost always produce complete songs. On the face of it, one might conclude that song itself is indivisible and unitary. However this conclusion is unwarranted, because it has been observed that zebra finches also produce a small number of variant songs in which certain syllables are transposed or dropped (Sossinka and Boehner 1980). Moreover, a previous study observed that zebra finches learn songs in syllable units, creating their songs by copying syllables or groups of syllables from adult males (Immelman 1969). It has been further reported that zebra finches can be induced to stop singing in the middle of song without invasive perturbation in the brain. Furthermore, the manner in which the singing was stopped indicated that the motor program for song contained discrete syllabic elements (Cynx 1990). These studies suggest that adult song remains somewhat plastic, to the extent that song syllables as unitary elements within the neural representation can be interrupted by intention or external perturbation. However, previous studies have reported that unilaterally stimulating HVC in an adult zebra finch during singing can often lead to syllable truncations (Vu et al. 1994; Ashmore et al. 2005; Wang et al. 2008) indicating that sub-syllabic units can represent smaller elements in birdsong.

Electrical stimulation is an acute experimental technique which invasively perturbs the neural activity and is likely to arbitrarily interrupt ongoing neural process. In order to
dissect a complex song into single behavioural units using noninvasive and natural stimuli, we performed a real-time, song-triggered, light-off experiment because songbirds usually do not sing in the dark. We investigated how putative elements of birdsong were interrupted by a bird’s reluctance to sing in the dark.

We further analyzed the data from previous electrical stimulation experiments (Wang et al. 2008) to check whether the stimulation induced syllable transitions can also be observed under normal conditions. Such transitions between behavioral units may imply that the song system is formed by a chain of sub-networks encoding each song unit. The distribution of effective chain lengths can also be inferred by inspecting the HVC stimulation effectiveness to provide us some insight about how such chain networks are likely organized. Our results clarify that the motor program for complex sequence learning and generation indeed acts on the sequential processing of elementary behavioural units.

7.2 Results

Instead of using acute strobe flashes (Cynx 1990), here we report the first use of a more natural visual perturbation for the purpose of inducing song suspension by brief song-triggered darkness for 100 ms. We found that ongoing birdsong could be suspended by presenting 100-ms darkness to the 4 birds that we used in total. We show the perturbation results from two birds in Figure 7.1 and 7.2.

In Figure 7.1, the sound-amplitude stack plot clearly demonstrates that songs are suspended by brief darkness (Figure 7.1A) with a peak latency of 133.8±6.1 ms (Figure 7.1B) that is about 60 ms longer than latencies to electrical HVC stimulation [see Chapter 3 or (Wang et al. 2008)]. This might be due to the extra time required for the detection of darkness via the visual pathways (Gonzalez et al. 2001; Romero et al. 2007). Inspection of the sound-amplitude stack plot reveals that a majority of song truncations occurred at the early part of silent intervals between syllables. Although this is consistent with a previous study that used more acute visual stimuli (Cynx 1990), we found that the bird’s song was also interrupted in the midst of the syllable, as depicted by the colored dashed arrows in Figure 7.1A. Even for the second example in Figure 7.2, in which most song truncations are narrowly locked to the inter-syllable intervals, we still observe several trials containing syllable truncations among most types of syllables (see colored dashed arrows in Figure 7.2A). Surprisingly, in this bird, although the peak truncation latencies for each syllable type are more variable (125.3± 24.7 ms), such latencies are still not significantly different from latencies in the first bird in Figure 7.1 (KS test and Student’s
test, \( p>0.1 \)). This might imply that the neural mechanism underlying darkness-induced song interruptions may be universal for zebra finches.

Figure 7.1. Brief startling visual stimuli lead to spontaneous song truncations (Example I).

(A) Top: spectrogram of a song bout from an adult male zebra finch “g1r3” with song motif indicated by the horizontal arrow. Middle: sound-amplitude stack plot depicting stimulation effects observed over the course of a day. Trials are ordered by onsets of stimuli (100 ms darkness), marked by white dots. The top ~400 traces depict nonstimulated catch trials, revealing the high stereotypy of song motifs. The dots of each color represent the truncation points in each type of syllable. Most of the song truncations occurred at the boundary of the syllables, but in this bird, there are also significant numbers of truncations in the midst of syllables (see indication by dashed arrows). Bottom: histogram of the truncation time with the dashed
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arrows indicating several numbers of truncations occurred in the midst of each type of syllable. (B) Histogram of truncation latency (time relative to stimulation onset) for each syllable type with the same color code used in (A). The peak latencies for each syllable type are very close to a fixed value (133.8± 6.1 ms).

Figure 7.2. Brief startling visual stimuli lead to spontaneous song truncations (Example II).

(A) Top: spectrogram of a song bout from an adult male zebra finch “o3r3” with song motif indicated by the horizontal arrow. Middle: sound-amplitude stack plot depicting stimulation effects observed over the course of a day. Trials are ordered by onsets of stimuli (100 ms darkness), marked by white dots. The top ~80 traces depict nonstimulated catch trials, revealing high stereotypy of song motifs. The dots of each color represent the truncation points in each type of syllable. Bottom: histogram of the truncation time with the
dashed arrows indicating several truncations occurred in the midst of each type of syllable. In this bird, most of the truncation points are around the end boundary of syllables. (B) Histogram of truncation latency (time relative to stimulation onset) for each syllable type with the same color code used in (A). The peak latencies for each syllable type are more variable than Figure 7.1B but are not significantly different (125.3 ±24.7 ms, KS test and Student’s test, p>0.1).

We inspected the spectrogram of truncated syllables to check if any special sound structure existed that was interrupted in the midst of the syllable. As shown in Figure 7.1A, many green-labeled truncations occurred in the midst of that syllable. Figure 7.3A displays such a truncation in the spectrogram with greater spectral detail. The spectral characteristic of this truncated syllable displayed a salient “down-sweep” (downward frequency-modulated harmonics) structure. In addition to this bird, we also found another bird whose song motif contained a similar “down-sweep” syllable that could be interrupted in the midst (see Figure 7.3B).

![Figure 7.3. Syllable with “down-sweep” spectral structure can be interrupted in the midst of it.](image)

(A) Spectrogram of the song motif sang by the same bird used in Figure 7.1. The top one is perturbed by a brief darkness at the time indicated by a red vertical line. The syllable interrupted by the perturbation is marked by the yellow dashed rectangle in which the white arrow indicates the syllable truncation point. The bottom spectrogram represented a non-perturbed catch trial. (B) Another bird sang a similar down-sweep syllable which can also be interrupted by brief darkness perturbation. Same caption as (A).

We have learned from our electrical stimulation results that inhomogeneity in birdsong circuits exists across hemispheres and is coordinated by some putative switching dynamics [see Chapter 3 or (Wang et al. 2008) for details]. Can such inhomogeneity also be revealed by our darkness perturbation that affects the song motor program via a totally different pathway, say visual pathway in this case? In Figure 7.4,
we show that for the same bird used for Figure 7.2, the song truncation susceptibility to darkness varied for different parts of the song. Before doing the darkness perturbation experiment on this bird, we performed HVC stimulation and showed that this bird indeed had such interhemispheric inhomogeneity for song production as shown in Figure 7.4A. In Figure 7.4B, the red dashed rectangles depicted the song parts that are much less interrupted by darkness perturbation. This suggests that although the perturbation was delivered into the song system indirectly via the visual pathways, once the signal entered into the song circuits, the song motor program may be affected by the same mechanism as that from the direct electrical stimulation. Nevertheless, we found that such effectiveness induced via the visual pathways can be adapted. As shown in the right panel of Figure 7.4B, the perturbation effectiveness 4 days later was saliently reduced but the CC between the LE curve and that from the first day is 0.30 that implies a qualitatively similar neural mechanism underlying the song interruption.

Our results have shown that zebra finches can be induced to stop singing in the midst of syllables and during the inter-syllable intervals by presenting brief darkness. The manner in which this stimulus stopped singing indicates that the song motor program contains discrete elements. We further hypothesized that the transitions between these unitary elements are probabilistically controlled in a repertoire of sub-networks that encode these elements. To verify our hypothesis, we manually inspected our previous stimulation data (see Methods) from right HVC stimulations to label all stimulation induced syllable transition and then went through every catch trial to check whether we could detect these transitions as well.
Figure 7.4. Darkness induced song truncations can be of different susceptibility. 

(A) HVC stimulation results before the visual perturbation experiment showed that when the stimulation amplitude is below a threshold (200 μA, single biphasic pulse with 0.2 ms per phase for this case), the distribution of song suspension is not homogeneous, of discrete contiguities, and just disjoint between two hemispheres [see Chapter 3 or (Wang et al. 2008) for details]. (B) The darkness perturbation results showed that some parts of the song can have different interruption susceptibility to the perturbation. As indicated by dashed red rectangles, songs are much less interrupted by the darkness. The right panel is the perturbation results 4 days later than the left panel and the LE curve showed that the effectiveness saliently reduced possibly due to adaptation. The CC between two LE curves is 0.30 that means despite the reduction of effectiveness over time, the neural mechanism underlying the song suspension remained similar.
Figure 7.5. Syllable transitions induced by electrical stimulation can be observed in the unperturbed condition.

Top most panel showed spectrograms of a normal song motif of this adult zebra finch that normally consisted of four syllable types labeled as number 1, 2, 3, and 4. But the bird also sometimes uttered two
extra syllable types labeled as 5 and 6 with much weaker stereotypy. Each of the rest panels comprises two spectrograms in which the top one represents a trial of right HVC stimulation and the bottom one represents a catch trial without any stimulation. Red vertical lines indicate the stimulation point in the ongoing song and the numbers indicate a syllable transition (e.g. “3 1” means transition from syllable 3 to 1 whereas a single number means that song stopped at the syllable labeled by that number). All of the labeled syllable transitions are collected from the data in the course of a day on 14th Sept. 2006. Asterisks indicate that the transition types were not found in the catch trials on the same day, but just found in some other days (see also in Figure 7.5). All spectrograms share the same axis properties with the top most spectrograms.

![2006-09-14](image)

**Figure 7.6. Histogram of electrical-stimulation induced and spontaneous syllable transitions.**
The digits code for syllable transitions in the same convention as in Figure 7.5. Red bars represent the number of stimulation-induced syllable transitions whereas the blue bars represent the number of spontaneous syllable transitions observed in catch trials. The histogram is computed from the data over the course of the same day as shown in Figure 7.4, but two transition types in catch trials as indicated by the black arrows were observed from different days shown as blue dates.
Figure 7.7. Most syllable transitions induced by electrical stimulation can be observed in the unperturbed condition.

Top most panel showed spectrograms of a normal song motif of this adult zebra finch that normally consisted of four syllable types labeled as number 1, 2, 3, 4, and 5. The top spectrogram of this panel
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showed a typical song motif with 5 syllables in which syllable 2-3-4 was usually repeated twice. The middle spectrogram showed that rarely the silent interval between syllable 4 and 5 was at least twice longer than average, so we labeled such abnormal silent intervals between syllable 4 and 5 as 6. Because zebra finches can control both side of vocal organs independently, the bottom spectrogram showed that this bird rarely single syllable 4 and 5 “simultaneously” that the corresponding syllable spectrograms overlap. We labeled such case as syllable transition type 7. All of the stimulation induced transition types are observed from the data in the course of a day on 16th Oct. 2005. Asterisks indicate that the transition types were not found in the catch trials on the same day, but just found in some other days (see also in Figure 7.7). The bottom most panel showed the only transition type that has never been found in catch trials from the courses of all experimental days. All spectrograms share the same axis properties with the top most spectrograms. Stimulation data were obtained from right HVC stimulation experiments.

![Figure 7.8](image)

**Figure 7.8. Histogram of electrical-stimulation induced and spontaneous syllable transitions.**
The digits code for syllable transitions in the same convention as in Figure 7.7. Red bars represent the number of stimulation-induced syllable transitions whereas the blue bars represent the number of spontaneous syllable transitions observed in catch trials. The histogram is computed from the data over the course of the same day as shown in Figure 7.6, but four transition types in catch trials as indicated by the black arrows were observed from different days shown as blue dates.

In Figure 7.5, we demonstrated that from 2,576 song motifs of an adult zebra finch recorded over the course of a day, 10 out of 12 transition types following stimulation were observed in catch trials on the same day, although less frequently (see figure
Surprisingly, the two exceptional transitions types were found just once in catch trials on rest of the experimental days (11 days). Figure 7.6 shows that the stimulation-induced transitions tend to occur with higher probability.

Figure 7.7 and 7.8 display another adult zebra finch with 15 types of syllable transitions in 1,289 stimulated song motifs over the course of a day. Notice that in this bird, there is one transition type we cannot find in over 8 experimental days (see the bottom most panel in Figure 7.7). However, bear in mind that two exceptional transition types in the first bird were just found with only one occurrence per type over 11 days, it might be likely that this transition was too rare to be observed within a limited number of days.

What is the possible neural circuitry mediating the syllable transitions? Previous studies have shown that, whereas RA neurons encode moment-to-moment patterns directly involved in generating the acoustic features of the song, HVC\textsubscript{RA} neurons encode the sequence and timing of these features (Hahnloser et al. 2002; Kozhevnikov and Fee 2007). Therefore, we consider that the network of HVC\textsubscript{RA} neurons may generate probabilistic syllable transitions forming variable syllable sequences. Our stimulation results may be due to direct stimulation (electrical stimulation) or indirect perturbation signals coming from visual pathways (darkness perturbation) acting on a chained HVC\textsubscript{RA} sub-networks encoding each unitary song elements. Figure 7.9 is a histological image to show that HVC\textsubscript{RA} neurons seem to be homogeneously and densely distributed within HVC. According to the simple current-distance relation (Ranck 1975; Tehovnik 1996), our electrical stimulation amplitudes ranging between 100 \( \mu \)A and 1 mA can possibly affect any combination of sub-networks. This might be why even some very small probability of special sequence transitions as shown in Figure 7.5 and 7.7 can be amplified by electrical HVC stimulation.

Chapter 3 and (Wang et al. 2008) reported that brief electrical perturbation of HVC can degenerate ongoing songs, with effects ranging from brief transient syllable distortions to complete song disruptions. The effectiveness of stimulation can be quantified by the percentage of time bins following stimulation in which song amplitudes deviate significantly from nonstimulated catch trials. It was found that for fixed-amplitude HVC stimulation, the effectiveness with which songs are distorted is highly modulated over the time course of a song motif, with highly effective and completely ineffective stimulation times following each other in short intervals (see Figure 7.10A as an example).
Figure 7.9. Sagittal view of HVC<sub>RA</sub> projection neurons labeled using fluorescent tracers.

The brain slice was of 50 µm thickness (top) and ~1.6 mm lateral from the sinus bifurcation (midline). All labeled HVC<sub>RA</sub> neurons are colored in red and are shown in higher magnification on the bottom. The yellow dashed line depicts the boundary of HVC. (The images were kindly provided by Moritz Kirschmann and Daniele Oberti.)
Furthermore, stimulation effectiveness tends to be complementary across the two cerebral hemispheres, such that low-amplitude stimulation disrupts songs when applied to one hemisphere but not the other. We interpret these findings as evidence of distinct chains formed by the HVC_{RA} neuron population. Accordingly, a putative HVC_{RA} chain corresponds to a contiguous set of highly effective stimulation times. We analyzed the stimulation data from \( n = 24 \) birds subjected to random HVC stimulation during singing as reported in Chapter 3 or (Wang et al. 2008). We inferred HVC_{RA} chain lengths from the time intervals between consecutive threshold crossings of stimulation effectiveness curves (see the red rasters in Figure 7.10A). The inferred distribution of chain lengths could be reasonably well fitted by a power-law function as shown in Figure 7.10B. We found that for a wide range of effectiveness thresholds, the scaling exponent of the best fit was close to \(-1\) (Figure 7.10C). These results have been used to support the scale-free chain-length distribution from the simple model in which HVC_{RA} chains develop as random permutation matrices (Fiete et al. 2010).

In summary, our results in this chapter make clear that song motor program contained discrete elements revealed by non-invasive visual perturbation that song can be interrupted either mainly on the syllable boundaries or less frequently in the midst of the syllable. Furthermore, subtle syllable transitions induced by HVC stimulation can also be observed in non-stimulation catch trial that suggests an underlying repertoire of HVC_{RA} sub-networks encoding each unitary song element. An HVC_{RA} chain can be inferred from a contiguous set of highly effective stimulation times and was formed to follow a scale-free chain size distribution, resembling many other real-world networks (Barabasi and Albert 1999), including the human brain (Eguiluz et al. 2005).
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Figure 7.10. Putative HVC_{RA} chains follow a scale-free size distribution.

(A) Example HVC stimulation. The spectrogram of a zebra finch song motif is shown on top. Electrical stimulation of HVC (0.2 ms biphasic current pulse of 500 μA) has a highly variable effectiveness as a function of stimulation time on ongoing singing (bottom, black line). HVC_{RA} chain lengths (horizontal red rasters, bottom) are inferred from periods of supra-threshold effectiveness (threshold 0.12, red horizontal line). (B) Distribution of inferred HVC_{RA} chain length from HVC stimulation experiments (circles, right and left HVC stimulation, n=24 birds, effectiveness threshold=0.12). The scaling exponent of the best power-law fit is -1.03 (red curve). A fit with scaling exponent -1 is shown for comparison (green curve). (C) Scaling Exponent of the optimal fit as a function of the effectiveness threshold. For a large range of thresholds, the scaling exponent is in the close vicinity of -1.

7.3 Discussion

Zebra finches were induced to stop singing by rapid switching off the light for a tenth of a second in the midst of song. The stimulation results are partially consistent with a
previous study that used acute visual stimuli (bursts of strobe light) to induce song suspension (Cynx 1990). The qualitative consensus from both studies is that even without invasively stimulating brain circuitries, song can be stopped in a discrete manner such that song suspensions almost always occur during the silent intervals between syllables.

It has been thought that production of vocal sequences by zebra finches is mediated by a “central motor program” (Konishi 1965; Konishi 1985; Vu et al. 1994). Our results suggest that syllables could be the unitary elements for which the central motor program engages ongoing monitoring and control mechanisms. Moreover, our results for the first time demonstrated that, similar to what we have found from invasive electrical stimulation in the brain (Wang et al. 2008), certain parts in the midst of syllables can also be suspended by sudden darkness. Furthermore, certain parts of the song are idiosyncratically resistant to perturbation, such that birds simply ignored the darkness and continued singing. The ability to stop song and maintain silence may confer an evolutionary advantage that a singing bird, startled by potential danger (ex. a rapid dark shadow underneath the flying predator’s open wings), can presumably increase its chances of survival by immediate silence.

Nevertheless, why did we not observe stopping during certain parts of the song? We speculate that these parts of song may be robustly encoded in the neural networks through versed practice during song development and thus once the central motor program is initiated to execute the neural codes for these parts, it is resistant to external perturbations. This would be an evolutionary tradeoff for male birds that sing more stereotyped songs, increasing their chance of courtship success (Searcy 1992).

Our results also showed that there are many noticeable exceptions that reveal sub-syllabic song suspension called “syllable truncation,” typically observed from electrical stimulation experiments. More specifically, some preliminary results demonstrated a tendency that syllables with downward frequency-modulated spectral features (down-sweeps) were vulnerable to darkness perturbation, causing the bird to stop singing in the midst of that syllable. These results contradict the conclusion that syllables are the smallest motor unit of song (Cynx 1990).

In order to resolve this puzzle, a recent theoretical model for song production (Gardner et al. 2001) should be mentioned here. This model of song production suggests that acoustically complex syllables might be produced simply by controlling variations in the frequency and relative phase of two driving parameters represented by the respiratory pressure and the elastic properties of the vibrating sound source in the vocal organ. This model demonstrated that a significant portion of syllable diversity could be achieved by varying the temporal relationship between the respiratory and syringeal components of
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the song motor programs. Synthetic signals that are qualitatively similar to a variety of natural syllable types, such as the complex harmonic sweeps, could be generated by varying the frequency and relative phase of these two parameters.

An implication of this model is that if we are to map central motor programs onto peripheral motor actions during song, it may be necessary to correlate activity in the central motor pathway with the proposed control parameters - that is respiratory pressure and the activity of syringeal muscles controlling the position and tension of the labia - rather than with the acoustic properties of the emitted vocalization (Suthers and Margoliash 2002). Our findings of frequent stimulus-induced song suspension in the midst of spectral down sweeps suggests that the central motor program controlling pressure and syringeal muscles during “down-sweep” sounds is specifically vulnerable to our startling perturbation (darkness). Additionally, inter-syllable interruption induced by strobe flashes (Cynx 1990) and our startling darkness may be due to the ease of interruption during respiratory intervals (Franz and Goller 2002).

Inspired by the model proposed by Gardener et al. (Gardner et al. 2001), one can speculate that song development may be a process that consists of a series of synaptic modifications among sub-networks which control the driving parameters represented by the biophysical properties such as the respiratory pressures and elasticity of labia. Such a neural process is idiosyncratic [see also discussion in Chapter 6 and (Wang et al. 2008)] to the extent that in the early learning stage, random sensory sub-networks which respond to parts of the tutor song (Williams and Nottebohm 1985) would serve as a template for the ongoing modified motor programs. When these networks produced song elements similar to the template, they would be rewarded (Doya and Sejnowski 1998; Fiete et al. 2007). Thus, the final central motor program will gradually form a repertoire of many sub-networks executed sequentially as a synfire chain during song production (Fiete et al. 2007; Jin et al. 2007). Any part (sub-network) of the central motor program can be perturbed to result in song alternation due to interruptions caused by internal neural noises (Rokni et al. 2007) or external stimuli such as electrical stimulations (Vu et al. 1994; Ashmore et al. 2005; Wang et al. 2008) and the startling darkness stimulus reported here.

Our HVC stimulation results indicate that electrical stimulation seems to selectively increase the likelihood of occurrence of some rare syllable transitions that existed in the putative syllable-transition repertoire, suggesting that variable syllable sequences may be generated in syllable-encoding chain networks connected into a probabilistic synfire chain pattern. During song production, the sequence of syllables is produced through a sequential chain of probabilistic synaptic events. The learned song
motif is most likely to be generated in the corresponding chain of sub-networks, whereas small probabilities still exist for some different sequences (rare syllable transitions).

A recent experimental study of reverse inactivation of right HVC following bilateral LMAN lesion showed that syllables can gradually be recovered from first to last (Thompson 2008). This result indicates an underlying neural representation that relies on a sequential chain of synaptic process in birdsong.

Meanwhile, another recent study in computational modeling suggested that syntax of birdsong syllable sequence is embedded in the connection patterns of HVC\textsubscript{RA} projection neurons (Jin 2009). Jin’s model proposed that variable syllable sequences are generated through spike propagations in branching chain networks in HVC. This model can be applied to qualitatively explain our findings about some nonlinear timing aspects between the stimulation times and the early song restarts [see text and Figure 3.2D in Chapter 3, and see also (Wang et al. 2008)]. When the sequence of syllables which is being processed sequentially in chained sub-networks is perturbed by electrical stimulation delivered at a certain node, the latencies for spikes to propagate to the node for song restart can highly depend on connection topology of the chain networks. Figure 7.11 illustrates a toy chain networks with certain network topology such that the latencies of song restarts are not necessarily constant, but such that the earlier stimulation can cause the later song restart (see figure caption for details).

In order to further understand how such chained sub-networks are organized, we inspected HVC stimulation effectiveness and found evidence for a scale-free size distribution of HVC\textsubscript{RA} sub-networks. The scaling exponent of roughly -1 that provided optimal fits to the data agrees with a recent model in which HVC\textsubscript{RA} chains develop as random permutation matrices (Fiete et al. 2010). Although this model does not invoke high-level mechanisms for the wiring of sequence-generating networks in HVC, there is evidence for such organization in terms of the temporal complementarity of HVC sub-networks across the two hemispheres. It remains to be seen whether this model can be appended by a simple competitive mechanism between the two cerebral hemispheres to explain these findings.
Figure 7.11. Simple HVC<sub>RA</sub> chain network model can explain the nonlinear timing aspects of the song motor program.

(A) An example in which HVC stimulation over a broad temporal range leads to identical syllable truncation times. In the unperturbed spectrogram (catch trial, top) we labeled the syllable types by 1, 2, 3, and 4 and also marked the normal offset time of syllable 2 with the magenta vertical dashed line. Below are song
spectrograms with identical syllable truncation times (pink vertical dotted lines) for both early and late stimulation (stimulation times are marked by white vertical lines). The sound amplitude stack plot (between the stimulation examples) reveals that all syllable truncations (pink dots, shown only for intermediate stimulation times) are vertically aligned, irrespective of stimulation time (white ramp dots, left). Note also that syllable truncations were followed by syllable 4 that was never observed at this location during catch trials. The onsets of this appended syllable are marked by white vertical dashed lines in the ‘early’ and ‘late’ stimulation examples and by the cloud of white dots in the stack plot (shown for intermediate stimulation times). An F-test revealed that the onset times of this appended syllable had the same variance when measured relative to song detection time and relative to stimulation time ($p=0.1$). Hence, the timing of this syllable was neither fixed to stimulation time, nor to song time. The red arrow in the sound amplitude stack plot indicated that the later stimulation time resulted in a fixed syllable truncation point and an earlier initiation of syllable 4 relative to that caused by earlier stimulation time indicated by the green arrow. (B) We illustrated a simple HVC$_{RA}$ chain networks that can qualitatively interpret the cause of different stimulation latencies indicated by the red and green arrows. Each syllable (1, 2, 3, or 4) is encoded by a HVC$_{RA}$ chain network. Spike activity during singing propagates from chain 2 to either chain 3 or 4, but not both. From our previous observation, the probability $P_{2\rightarrow3}$ for transition from syllable 2 to 3 is 100% during catch trials, but the probability $P_{2\rightarrow4}$ for transition from syllable 2 to 4 overtook $P_{2\rightarrow3}$ when stimulating HVC during syllable 2. Synaptic connections from chain 2 to chain 4 are colored in blue. All black arrows represent the synaptic pathways in catch trial. When stimulating at the early part of syllable 2 indicating by the big green arrow, the stimulation current interrupts the spiking activity all up to the neurons marked by solid pink circles due to the stimulation-induced interference with certain external input controlling syllable offsets. This will thus result in a fixed syllable truncation time as also depicted by the pink dots accordingly in (A). The abstract truncation latency in this toy model is the time equivalent to 4 monosynaptic conductions (4 layers of green arrows). Meanwhile, the stimulation also triggered the synaptic connections (blue curved arrows) linking all the way down to the neurons (solid white circles) in chain 4 to generate syllable 4 with onsets depicted by white dots accordingly in (A). The latency of this onset is equivalent to 5 monosynaptic conductions (5 layers of blue arrows). The same caption is also used for the case of stimulation at the later part of syllable 2 indicating by the big red arrow and thus the syllable-2 truncation latency and the syllable-4 onset latency are equivalent to 2 and 3 monosynaptic conductions, respectively.

More experiments are necessary to answer some issues raised from the above discussions, such as how does the neural system maintain the stability of such embedded synfire chains (Li and Greenside 2006) and how can hardwired sub-networks survive from neurogenesis in adult HVC.
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7.4 Methods

**Song-triggered darkness.** We used the same real-time song detection system as described in chapter three. Once a song motif was detected, a 5V TTL pulse was generated to drive a mechanical relay (Power PCB Relay/RT114006, Tyco Electronics, CA, USA) to turn off the light for 100 ms in the sound-proof chamber. This resulted in the bird to stop singing for the short period of darkness.

**Categorization of syllable transitions.** To pick syllables, for each bird we first chose a sound-amplitude threshold slightly above cage-noise level (we found that a threshold of 20% into the 1-99th percentile interval worked well for all birds). For all song motifs from each bird, under visual supervision, we identified a syllable by taking the intervals between consecutive threshold crossings of song intensities (first crossing from below and second crossing from above). For selected syllables, we manually labeled each syllable with a number corresponding to a syllable type by visually inspecting the spectrograms. All of the manual procedures are done with the aid of computer graphical user interface implemented in Matlab (Mathworks, USA).

**Analysis of song truncations.** First we measured baseline distributions of syllable lengths from data of selected catch trials (only complete motifs). Syllables were identified using the method of threshold crossing described above. Stimulated syllables were then classified as truncated if their duration was within the lowest percentile of the baseline distribution. For darkness-induced song truncations, we searched for truncations in a time window up to 507 ms (corresponding to 130 time bins of 3.9 ms or 128 sound samples each) after stimulation.

**Analysis of putative HVC_{RA} chain length.** Stimulation data was analyzed as described in Chapter 3 or (Wang et al. 2008). Briefly, for each discrete stimulation time, we tested whether the sound amplitudes in 3.9-ms bins after stimulation were different from amplitudes in matched time bins during catch trials using the Kolmogorov-Smirnov (KS) test ($p=0.01$). For each set, we quantified the stimulation effect by the fraction (percentage) of time bins in which significant differences were detected. The late-effect curve in Figure 7.9A was based on bins ranging from 78 to 312 ms after stimulation (bins 21 to 80). We inferred the putative HVC_{RA} chain lengths from the time intervals between consecutive threshold crossings of stimulation effectiveness curves (first crossing from below and second crossing from above). The power-law curve fitting for chain size
distribution was done by using Matlab function `fit.m` with input of base function $x^r$ defined in `fittype.m`. 
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Appendix A

LabVIEW FIFO design pattern for the real-time song-triggered stimulator/recorder

Figure A.1. LabVIEW block diagram of the basic architecture for real-time song-triggered stimulator via the data acquisition (DAQ) card.

The key element to implement a real-time system for song-triggered stimulator or recorder is the design pattern “First In, First Out” (FIFO) that is up to now one of the most popular computerized paradigm for real-time systems. In ways, it is an abstraction of organizing and manipulating data relative to time and prioritization. This expression describes the principle of a queue processing technique that means what comes in first is handled first, what comes in next waits until the first is finished, etc.

Our general-purpose FIFO real-time system consists of three major components which are all linked by two FIFO queuing data structures. The whole real-time procedure starts from the first component for the control of continuous analog input on a multifunction data acquisition (DAQ) card (PCI-6251, National Instruments, TX, USA) that acquires a fixed number of samples (read-in buffer) and then immediately passes them through the FIFO data queuing pipeline to the second component for real-time data processing implemented for song detection (see Methods in Chapter 3 for details). The results of the data processing is wrapped as a chunk of data of the same length as the
read-in buffer (128 samples in our design) and is immediately sent to the third component through a separate FIFO data queuing pipeline for continuous analog out on the same DAQ card. Notice that for analog input, analog output, or data processing, all tasks are running continuously within the multi-threading while-loops in LabVIEW. The data flow thus will be seamlessly processed in real-time with totally only a fixed delay time $\tau$ required for acquiring data into the read-in buffer [$\tau = 128 \text{ samples} \times 30 \mu\text{s (sampling rate)}$] plus the CPU time required for data processing and the tiny overhead of LabVIEW for data transmission among components. We make sure that the data processing time is less than $\tau$, otherwise the queuing pipeline will accumulate unprocessed data and thus all components will be out of synchrony (i.e., no longer in real-time).

For the song-triggered stimulator, it is sufficient to use the three-component (analog input, data processing, and analog output) system in which the analog output component will be used for delivery of stimuli. However, for the song-triggered stimulator with an extra data saving function, we need to implement one more component to save data, sharing the same data queuing pipeline linking between the data processing and analog output components. In this system, the data processing component will, according to some pre-defined rules, determine whether to pass the pre- and/or post-processed data to the data saving component for saving the data into a hard drive. Normally the part for data saving does not need to be in real-time that can make the system less CPU intensive.
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07/2003-10/2003 Associate of IT Division of CERN, Geneva, Switzerland as a member of the Grid Technology Area Group under CERN LHC Computing Grid (LCG) project.
08/2003-09/2003 Student at CERN School of Computing 2003 by CERN and Donau University, Krems an der Donau, Austria.
08/1998-09/1998 Summer Student in Physics of Molecular Biology, Summer School by NORDITA and the Niels Bohr Institute, University of Copenhagen, Denmark.
Teaching


09/2005-02/2006 Teaching Assistant in the graduate course “Biophysics of Neural Systems” at ETH Zürich.

Publications


Curriculum vitae


Conferences


