Title:
Sleep-related spike bursts in HVC are driven by the nucleus interface of the nidopallium

Abbrev. Title:
NIf drives bursts in the sleeping songbird

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Content:
2 Tables, 7 Figures, 31 pages

Keywords:
zebra finch, sequence, efference copy, birdsong, neural code, antidromic

Acknowledgements:
We thank Maria Minkoff and three anonymous reviewers for comments on the manuscript.

Grants:
These studies were supported by a Swiss National Science Foundation grant to R.H. and National Institute of Mental Health Grant RO1-MH067105 to M.S.F.
ABSTRACT

The function and the origin of replay of motor activity during sleep are currently unknown. Spontaneous activity patterns in the nucleus robustus of the arcopallium (RA) and in HVC (a letter-based name) of the sleeping songbird resemble premotor patterns in these areas observed during singing. We test the hypothesis that the nucleus interface of the nidopallium (NIf) has an important role for initiating and shaping these sleep-related activity patterns.

In head-fixed, sleeping zebra finches we find that injections of the GABA$_A$-agonist muscimol into NIf lead to transient abolishment of premotor-like bursting activity in HVC neurons. Using antidromic activation of NIf neurons by electrical stimulation in HVC, we are able to distinguish a class of HVC-projecting NIf neurons from a second class of NIf neurons. Paired extracellular recordings in NIf and HVC show that NIf neurons provide a strong bursting drive to HVC. In contrast to HVC neurons, whose bursting activity waxes and wanes in burst epochs, individual NIf projection neurons are nearly continuously bursting and tend to burst only once on the time scale of song syllables. Two types of HVC projection neurons, premotor and striatal projecting, respond differently to the NIf drive, in agreement with notions of HVC relaying premotor signals to RA and an anticipatory copy thereof to areas of a basal ganglia pathway.
INTRODUCTION

Studies of motor-related neural activity and their widely observed replay during sleep are of a broad interest as they relate to the natural mechanisms for encoding and consolidation of memories (Nadasdy et al., 1999; Fenn et al., 2003; Palchykova et al., 2006; Walker and Stickgold, 2006). Premotor activity in the songbird nucleus robustus of the arcopallium (RA) is correlated with song vocalizations on a temporal scale in the submillisecond range (Yu and Margoliash, 1996; Chi and Margoliash, 2001; Leonardo and Fee, 2005). RA neuron activity has also been recorded during sleep, where it strongly resembles song-related activity (Dave et al., 1998; Dave and Margoliash, 2000). Song and sleep-related spike patterns are believed to be generated by common neural mechanisms, since during both singing and sleep, RA spike patterns are driven by sparse sequences in HVC projection neurons (Hahnloser et al., 2002; Hahnloser et al., 2006). Given that HVC projection neurons are of such importance to RA activity in the singing and sleeping states, we speculate that projection neurons upstream of HVC are of similar importance to the generation of sparse HVC sequences. Here we are interested in elucidating the feedforward drive provided to HVC during sleep from the nucleus interface of the nidopallium (NIf).

HVC receives input predominantly from the thalamic nucleus uveaformis (Uva) in the thalamus, the medial magnocellular nucleus of the anterior nidopallium (MMAN), and NIf (Nottebohm et al., 1982; Fortune and Margoliash, 1995; Vates et al., 1996; Rosen and Mooney, 2006). NIf is embedded in the auditory field L complex and is believed to be important for auditory-motor interactions in the song-control system. Lesions of NIf have been found to abolish selectivity in HVC neurons to acoustic playback of the bird’s own song (BOS) (Janata and Margoliash, 1999; Coleman and Mooney, 2004; Cardin et al., 2005). Similarly, spontaneous activity in HVC of anesthetized birds is strongly reduced after reversible or irreversible NIf lesions (Coleman and Mooney, 2004; Cardin et al., 2005).

We are interested in elucidating the sleep-related activity patterns of NIf neurons projecting to HVC (NIf_{HVC} neurons). These NIf neurons were previously identified in anesthetized birds and found to be more densely firing during BOS playback than HVC
projection neurons (Coleman and Mooney, 2004). Our goal is to identify potential differences in the manner in which these NIf neurons drive activity patterns in downstream areas in the premotor and the anterior forebrain pathways (Okuhata and Saito, 1987) involved in song production and song learning (Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991; Doupe, 1993; Doupe et al., 2004). To characterize the relationship of sleep-related activity patterns in higher premotor brain areas, we study coherency functions from paired recordings of NIfHVC neurons and HVC projection neurons, and study the effects of reversible pharmacological inactivation on sleep-related spike trains. Our study reveals new insights into the dynamics of sleep-related activity patterns and provides the first evidence of a target-specific population code of NIf neurons. Parts of this paper have been published previously in abstract form (Fee et al., 2002).

MATERIALS AND METHODS

Our experiments were performed in head-fixed zebra finches, a preparation that permits us to perform paired extracellular recordings from identified neurons and pharmacological manipulations. General methods and procedures for the experimental setup of head-fixed, sleeping birds and spike train data analysis have been described previously (Hahnloser et al., 2006).

Subjects. Zebra finches (Taeniopygia guttata) were obtained from commercial suppliers (Old Bridge, NJ (USA) and Animal Diffusion, Villarimboud (Switzerland)). Animals were maintained on a day-night reversed 12 hour light cycle to assist in obtaining sleep during daytime experimental sessions. Data were taken from a total of 36 adult zebra finches (>90d).

Surgery. Surgery began approximately at the onset of the night cycle. Birds were anesthetized with 1-3% isoflurane in oxygen. A small hole (~200 µm) was made in the dura over each area and the uncovered brain was protected with 2% low melting agarose (Sigma, Inc). Bipolar stimulation electrodes (Teflon-insulated 50 µm diameter stainless steel wire spaced 0.5 mm apart) were inserted into HVC and RA or Area X and secured to the skull with dental acrylic. Wound margins were treated with lidocaine gel. The animal was placed in a small foam restraint and subsequently moved to the recording
apparatus without further anesthesia. In some of the experiments, to promote sleep, the bird was given a single dose (1-10 µg) of melatonin (Sigma) right after surgery, injected subcutaneously in phosphate buffered saline.

**Electrophysiology**

*Antidromic activation.* Bipolar stimulation electrodes in HVC were used for antidromic identification of NIf neuron type and RA (or Area X) stimulation electrodes were used for identification of HVC neuron type (Fig. 1A). Electrical stimulation was produced using an isolated stimulation unit (AMPI, Inc) with intensities varying from 50-500 µA. For most experiments, single monophasic current pulses of 0.2 ms duration were used as stimuli. Single or paired single-unit recordings were made in HVC and NIf. High signal-to-noise (>10:1) recordings were made using sharp glass microelectrodes (5–15 MΩ, borosilicate, 1.0 mm OD, 0.7 mm ID) pulled on a vertical electrode puller (Model 730, Kopf Instruments, Inc) and filled with 2 M NaCl. Signals were amplified using a Neurodata IR285 (Cygnus Instruments, Inc) or Axoclamp-2B (Molecular Devices, Inc) intracellular amplifiers. Custom electronics were used for additional amplification (gain of 100) and filtering (300 Hz high-pass 5-pole Bessel filter, 10 kHz low-pass constant delay filter). Extracellular signals were digitized to 16 bits precision at a sampling rate of 20 kHz and stored in blocks of 200 seconds on a Pentium-based PC running custom Labview software (National Instruments, Inc.).

*NIf identification:* For the NIf identification experiment in Figure 1B, we first pressure injected 200 nl of 10% biotinylated dextran amines (BDA) in phosphate-buffered saline into right HVC. The purpose of this tracer injection was to fluorescently label NIf neurons. Two days later, under anesthesia, we penetrated NIf from a posterior angle with a 1–3 MΩ platinum tungsten electrode. Every 20 µm between 2 and 3 mm penetration depth, we recorded the responses to 30 HVC stimulations at 1 second intervals (~200 µA amplitude). After assessing the extent of NIf by observing the stimulation responses on an oscilloscope, we made two electrolytic lesions above and below NIf, 900 µm apart. Lesions were made by injecting a constant current of 15 µA for approximately 12 seconds. These lesions were later identified in histological sections in order to precisely locate the electrode track within sagittal slices of the brain.
Stimulation response shown in Figure 1B was quantified by the root-mean squared (RMS) Voltage amplitude in the 1–7 ms interval following stimulation onset (averaged over the 30 stimulations per site).

*NIf neuron identification:* NIf neurons were found and isolated using ongoing 1 Hz stimulation in HVC to elicit spike responses. Once a cell was isolated, stimulation threshold current was measured and stimulus intensity was set between 10% and 50% above threshold to produce a reliable response. Continuous analog records were acquired for 60 stimuli at 1 second intervals. The response latency of an antidromic spikes is defined as the interval between stimulus onset and the first evoked spike. Latency variability is defined as the standard deviation of response latency. We estimate that the contribution to the latency variability due to our fixed sampling rate of 20 kHz is less than 16 µs (dashed line in Fig. 1E). Because neurons in neighboring field L may also project to HVC (Fortune and Margoliash, 1995), we tried to avoid recording from field-L neurons by recording only from those NIf regions with very dense multiunit response (hash) to stimulation, shown to correlate well with NIf extent (Fig 1B). Furthermore, we observed that field L neurons projecting to HVC (L_HVC neurons) had longer response latencies to HVC stimulation than did NIf_HVC neurons. Also, L_HVC neurons showed strong phasic responses to brief sound clicks, whereas NIf_HVC neurons did not. For the NIf neurons classified as interneurons, we were similarly conservative and applied this classification only to neurons found at penetration depths within the electrophysiologically identified borders of NIf.

*Reversible lesions in NIf.* The effect of lidocaine and muscimol injections in NIf on sleep-burst rate in HVC interneurons was examined by micro-injection of lidocaine or muscimol in 2% phosphate-buffered saline during single-unit recordings of HVC1 neurons. Injections were made from pulled glass pipettes (50 µm tip size) using a pressure injection system (Pico Spritzer, Inc.). Injected volumes were 10–100 nl. Control injections were made in NIf with phosphate buffered saline. Typically, injection pipettes were placed in the center of NIf after an electrophysiological recording session during which the extent of NIf was well characterized by multiple electrode penetrations. To measure the effect of the injection, we counted the number of bursts in the HVC1 neuron in the minute before and after the injection. Statistical significance of burst rate
differences before and after the injection was assessed using the Mann-Whitney U test (p<0.001). In one case the bird woke up right after the injection (by observation of eye opening). Because the burst rate in HVC neurons is known to be highly state dependent (Hahnloser et al., 2006), data from this trial were discarded.

**Histology:** After each experiment, the animal was euthanized by intramuscular injection of 20% urethane or nembutal. For the NIf identification experiment, birds were transcardially perfused with 4% paraformaldehyde (PFA) in 25 mM NaPO₄ buffer. The brain was removed for histological examination of Nissl-stained (or unstained) slices to verify the location of stimulating and recording electrodes, and drug injection sites. All experiments were carried out in accord with protocols approved by the local IACUC and the Veterinary Amt of the Canton of Zurich, Switzerland.

**Spike train Analysis.**

All spike train analysis procedures were implemented with custom-made Matlab (Matworks Inc., Natick MA, USA) scripts.

**Instantaneous firing rate:** One way of representing the activity at time $t$ of a neuron is by the instantaneous firing rate $R(t)$, a continuous function defined by the inverse of the interspike interval surrounding time $t$.

**Burst rate:** We identified a group of at least two spikes as a burst if the instantaneous firing rate continuously exceeded 100 Hz. The burst rate is defined as the number of events per unit time in which the instantaneous firing rate crosses the 100 Hz barrier from below.

**Interspike interval (ISI) probability density function (pdf):** For each neuron A we computed the ISI pdf $h_A(\tau)$ as a basic characterization of its spike train ($\tau$ stands for the ISI). First we computed the ISI histogram with bin centers $\tau_i$ chosen on a logarithmic scale ($i = 1, ..., 100$). The ISI pdf $h_A(\tau_i)$ is simply a normalized ISI histogram satisfying $\sum_i h_A(\tau_i) = 1$. 
Auto-covariance function: The auto-covariance function $C_{AA}(t)$ of a spike train $\rho_A(t)$ (modeled as a sum of delta functions) is a measure of spike density fluctuations. It is computed as

$$C_{AA}(t) = \frac{1}{T - |t|} \int_0^T \rho_A(s + t)\rho_A(s)ds - \bar{\rho}_A^2,$$

where $\bar{\rho}_A$ is the mean firing rate of neuron A, and $T$ is the duration of the recording (the denominator $T - |t|$ results in an unbiased estimation of auto-covariance at large times $t < T$). The first term on the right side of Equation (0.1) is known as the auto-correlation function. By subtracting the term $\bar{\rho}_A^2$ in Equation (0.1), the asymptotic value of the auto-covariance function is zero. For better visibility, we have plotted auto-covariance functions on a logarithmic time scale $t_i$ in Figures 3C and 3D by integration between adjacent points, i.e., plotted is the discrete function $C_{AA}(i) = \int_{t_i}^{t_{i+1}} C_{AA}(t)dt$.

To assess the complexity of spike trains, we probed them for renewal dynamics, i.e. we tested whether interspike intervals were generated by random sampling from the ISI pdf $h_A(\tau)$. Renewal spike trains have no memory, as each ISI is a random variable that is independent of the previous ISI. To perform this test, we have computed renewal auto-covariance functions $C_{AA}^R(t)$ derived from the ISI pdfs under renewal assumptions (here superscript ‘R’ stands for renewal), and compared these functions to the measured auto-covariance functions. For a derivation of $C_{AA}^R(t)$ see e.g. (Gerstner and Kistler, 2002). Any deviation of $C_{AA}(t)$ from $C_{AA}^R(t)$ is indicative of a memory in the spike train, causing ISIs to be conditional on previous ISIs. A deviation at time $t_i$ is significant if $C_{AA}^R(i)$ differs from $C_{AA}(i)$ by more than three times the standard error of $C_{AA}(i)$ (the standard error was computed by jackknifing - see the Methods section on the coherency analysis).

We also tested whether auto-covariance functions differed from renewal functions on the population level. Average auto-covariance functions $\langle C_{AA}(t) \rangle_A$ and $\langle C_{AA}^R(t) \rangle_A$ for the two NIf neuron types are shown by the full and dashed lines in Figures 3C and 3D. At
each time $t$, we tested whether the auto-covariance and renewal functions at that time had identical medians using the Wilcoxon rank sum test ($p<0.01$). Time intervals where deviations were significant are indicated by thick horizontal bars in Figures 3C and D.

**Coherency analysis:** We have quantified correlations between spike trains in simultaneously recorded cells by the coherency function $\gamma_{AB}(t)$, which is similar to the cross-covariance function, but includes an additional normalization by the auto-covariance functions of the two spike trains. This normalization serves to discount for correlations arising from bursting tendencies of neurons (Thomson and Chave, 1991; Kimpo et al., 2003). In the frequency domain $\omega$, the coherency function $\gamma_{AB}(\omega)$ is computed by normalizing the cross-covariance function $C_{AB}(\omega)$ between neurons A and B by the square-roots of the auto-covariance functions:

$$\gamma_{AB}(\omega) = \frac{C_{AB}(\omega)}{\sqrt{C_{AA}(\omega)C_{BB}(\omega)}}. \quad (0.2)$$

We determined the coherency in the time domain $\gamma_{AB}(t)$ by inverse Fourier transformation of $\gamma_{AB}(\omega)$. As in our previous study (Hahnloser et al., 2006), we smoothed the coherency function $\gamma_{AB}(t)$ by convolution with a Gaussian windowing function of 4 ms standard deviation. Finally, the smoothed coherency functions were down-sampled to 1 ms temporal resolution by summing over coherencies in 1 ms bins. Our finding do not critically depend on the parameter values for smoothing and down-sampling.

To depict the typical coherency function between neuron types, we have plotted the average coherency function $\left\langle \gamma_{AB}(t) \right\rangle_{AB}$, Figures 6C, 6E, 7B, and 7D.

**Significance:** The significance threshold for the coherency function of a particular neuron pair was assessed by jackknifing the data in 10 second data windows and computing the jackknife variance (Thomson and Chave, 1991)

$$\sigma_j^2(t) = \frac{N-1}{N} \sum_{i=1}^{N} \left( \gamma_{AB}^i(t) - \left\langle \gamma_{AB}^i(t) \right\rangle_{AB} \right)^2,$$

where $N$ represents the number of data windows (depending on the duration of the recording), $\gamma_{AB}^i(t)$ is the coherency function of the jackknifed data with the $i^{th}$ window
missing, and \( \overline{\gamma}_{AB}(t) = \langle \gamma_{AB}(t) \rangle \), is the average coherency function of the jackknifed data. We set the significance threshold of measured coherencies to \( 3\sigma_j(t) \), shown by dashed lines in Figures 6 and 7. This threshold corresponds to a roughly 99% confidence for the significance test. Whenever the coherency function exceeded the significance threshold, a peak emerged. The distributions of peak amplitudes versus peak latencies are shown in the summary plots beneath the average coherency functions in Figures 6C, 6E, 7B, and 7E.

We have also analyzed coherency functions on a population level, to see whether they had some characteristic shape. For all neuron pairs of given types, the average coherency function was compared to the average significance threshold shown by dashed lines in Figures 6C, 6E, 7B, and 7D. Characteristic peaks in average coherency were found by testing whether differences between the coherency functions and the significance thresholds had zero median using the Mann-Whitney signed rank test (p<0.01). Time intervals where the median coherency function was significantly larger than the median renewal function are indicated by thick horizontal bars in Figures 6C and 7D.

**RESULTS**

We set out to characterize the role of NIf input for generating and shaping sleep-related spontaneous activity in identified HVC neurons. As different types of NIf neurons have not been previously classified, and their firing properties and correlations with identified HVC neurons during sleep are unknown, we (1) distinguished NIf\(_{HVC}\) neurons from other NIf neuron types using antidromic activation by electrical stimulation in HVC, (2) analyzed spike train statistics of identified NIf neurons, and (3) assessed spike train correlations and coherencies of identified NIf and HVC neurons. Furthermore, to characterize the properties of population activity in NIf and assess its relevance for shaping spike trains in HVC, we (4) pharmacologically blocked NIf activity while recording spike trains from identified HVC neurons. All our recordings and manipulations were performed in the sleeping, head-fixed bird (see Methods). A
A schematic of the zebra finch brain showing NIf and HVC and the location of stimulation and recording electrodes is shown in Figure 1A.

**NIf identification**

NIf is surrounded by auditory areas of the field L complex (L1, L2, and L3). Since neurons in field L1 and L3 have projection targets in the shelf of HVC, one could imagine confounding them with NIf neurons when searching for antidromic responses to HVC stimulation. However, there exist cytoarchitectural and morphological differences between NIf and areas of the field L complex. Cytoarchitectural studies have revealed increased cell density in NIf in comparison to L1 and L3, and morphological studies have revealed differences in predominant Golgi cell types found in NIf and field L (Fortune and Margoliash, 1992; 1995). Based on these studies we expected to find increased density of extracellular responses to HVC stimulation when placing the recording electrode inside NIf.

By quantifying the multiunit response density (stimulation hash) by the root-mean-squared (RMS) voltage trace in a small time interval following HVC stimulation, we found indeed a robust increase in RMS voltage when the recording electrode was positioned within NIf (Fig. 1B). In this experiment, we verified the passage of the electrode track through NIf by examining histological sections of the brain in which NIf was retrogradely labeled by fluorescent indicator (see Methods). In the following experiments, we used the clearly visible increase in stimulation hash at short stimulus latencies as an operational assessment of NIf extent. All single unit recordings were performed at penetration depths within this antidromically characterized region.

**Classification of NIf neurons**

We identified two distinct NIf response patterns to HVC stimulation (Fig. 1C, D). Putative HVC-projecting NIf neurons responded to low-intensity stimulation (50-500 µA) in HVC with a single spike. Near-threshold stimulation produced latencies to the first spike between 0.9 and 3.2 ms (average 1.6±0.6 ms, n=20) with small latency variability in the range 14-69 µs (37±16 µs, n=20, Fig. 1E). Putative NIf interneurons responded to low-intensity stimulation in HVC, with an increasing number of spikes for
increasing stimulus intensity. Near-threshold stimulation produced latencies to the first spike ranging from 1.8 to 5.4 ms (mean 3.2±1.4 ms, n=5) and a large latency variability in the range 236–887 µs (559±317 µs, n=5, Fig. 1E). On the basis of these results, we infer that the neurons with small latency variability were HVC-projecting (NIfHVC) neurons. For simplicity, we refer to the neurons with large latency variability as NIf interneurons (NIfI neurons), although we cannot rule out that these neurons project outside of NIf. In agreement with this classification, in all cases tested (n=20), we found that neurons with latency variability less than 100 µs (putative NIfHVC neurons) exhibited spike collisions when stimulated immediately after a spontaneous spike (Fig. 1C bottom). Neurons with latency variabilities larger than 100 µs (putative NIfI neurons), on the other hand, did not exhibit collisions (Fig. 1D bottom). The vast majority of neurons isolated within the borders of NIf, as defined by the presence of antidromic hash, had small latency variability and had firing patterns as described below for NIfHVC neurons. Long latency variability neurons were rarely found, and all of these had firing patterns similar to those described below for NIfI neurons. In the subsequent experiments, we identified each recorded NIf cell by its antidromic stimulation response, distinguishing NIfI neurons from NIfHVC neurons by their differences in spike latency variability.

The spike widths, measured at 25% of the peak amplitude, were smaller for NIfI neurons (0.16±0.02 ms, range 0.13–0.19 ms, n=9) than they were for NIfHVC neurons (0.20±0.04 ms, range 0.13–0.32 ms, n=51, Fig. 1F). However, based on the considerable overlap between spike width distributions, we were unable to classify neuron type based on spike width or even spike waveform (using principal component analysis).

In many simultaneous recordings in NIf and HVC, we also identified HVC neuron type by RA stimulation. In the course of these experiments, we found two neurons in NIf (in two birds) that projected both to HVC and to RA, as judged by the spike collision test. However, we were not able to determine whether these neurons formed monosynaptic connections with both HVC and RA neurons; or whether the axonal pathways of these HVC-projecting neurons simply passed in close proximity to RA, but without targeting RA neurons. Note that RA has not been identified as a projection target of NIf neurons in the literature.
Spike train properties of two NIf neuron types

During sleep, a large fraction of spikes in both NIf neuron types fell into high frequency bursts (Fig. 2A,B). Nevertheless, NIf\textsubscript{HVC} and NIf\textsubscript{I} neurons differed considerably in their spontaneous firing patterns during sleep. For example, the average firing rate of NIf\textsubscript{HVC} neurons was 5.4 Hz, whereas that of NIf\textsubscript{I} neurons exceeded 27 Hz. NIf\textsubscript{HVC} neurons fired fewer bursts per second and exhibited larger average instantaneous firing rates during bursts than did NIf\textsubscript{I} neurons (Wilcoxon rank sum test, n=117 NIf\textsubscript{HVC} neurons and n=13 NIf\textsubscript{I} neurons, p<0.01). Average firing rates, firing rates during bursts, burst rates, burst widths, number of spikes per burst, and the fraction of total spikes that are part of bursts are summarized in Table 1.

In our previous recordings we found that firing rates in HVC and RA neurons waxed and waned, altering between a state of high firing lasting 1-2 seconds and a state of low firing also lasting several seconds (Hahnloser et al., 2006). We previously identified a suitable time scale to measure these firing-rate fluctuations to be 3 seconds. When analyzed in similar 3 second windows, we did not find similarly large fluctuations of firing rates in NIf neurons. Average firing rates of NIf\textsubscript{HVC} neurons were distributed in the range from 0 Hz up to 22 Hz (filled circles in Fig. 2C). This range of firing rates is comparable to that of HVC\textsubscript{X} neurons, a HVC neuron type of average firing rate less than a third of that of NIf\textsubscript{HVC} neurons. NIf\textsubscript{I} neurons rarely displayed firing rates less than 5 Hz or larger than 50 Hz (Fig. 2D). In comparison, HVC\textsubscript{I} neurons, despite having half the average firing rate, achieved about twice the range of NIf\textsubscript{I} firing rates. In Figures 2C and D, we also show firing rate distributions of NIf neurons in burst spike trains (spike trains for which all single spikes not part of a burst have been removed for the analysis), verifying that on a coarse time scale, small firing-rate fluctuations also applied to bursts.

This lack of extensive firing-rate fluctuations in NIf motivated us to measure spike train statistics in NIf\textsubscript{HVC} neurons also in awake birds, as judged by their open eyes after touching their tails (n=3 birds). In data from 3-minute recording sessions, we found that firing rates and burst rates of NIf\textsubscript{HVC} neurons in the awake state were not statistically different from the firing rates measured during sleep (Wilcoxon rank sum test, n=5
NIHVC neurons, p<0.01; Table 1). Note that in our previous sleep studies in HVC and RA, we never observed bursts in awake birds in any neuron type.

**Interspike-interval (ISI) and auto-covariance functions**

We decided to further study NIH spike trains and the biophysical mechanisms responsible for their generation by examining interspike interval (ISI) probability density functions (pdfs), and autocovariance functions. These functions are computed and analyzed in the following.

The average ISI pdf of each NIH neuron type is bimodal, with a common first peak corresponding to the ISIs during bursts and a second peak roughly corresponding to the interburst interval (Fig. 3A, B). The average ISI pdf of NIH_{HVC} neurons is reminiscent of that of HVC projection neurons, as these neurons also emit virtually no spikes at an ISI of about 10 ms (Hahnloser et al., 2006). Similarly, the average ISI pdf of NIH_{I} neurons is reminiscent of that of HVC_{I} neurons.

In HVC projection neurons there is evidence of intrinsic (not synaptically driven) bursting as judged by the stereotypy of recorded sleep bursts (Hahnloser et al., 2002), whereas in HVC interneurons and RA neurons, there is no such evidence (Dave and Margoliash, 2000). We investigated the burst stereotypy of NIH neurons by computing autocovariance functions. In 83 of 117 NIH_{HVC} neurons, we found an oscillatory behavior of autocovariance functions on a very short time scale, with an example shown in the inset of Figure 3C. This oscillatory behavior is a consequence of stereotyped burst patterns and suggests a cellular rather than a network mechanism for burst generation in NIH_{HVC} neurons. Only 1 of 13 NIH_{I} neurons showed an oscillatory auto-covariance function, thus providing weaker evidence for intrinsic bursting mechanisms in NIH_{I} neurons (a typical non-oscillatory auto-covariance function is shown in the inset of Fig. 3D).

The simplest generative process of a spike train with a given ISI pdf is the renewal process, according to which each ISI is drawn randomly and independently of other ISIs from a fixed ISI pdf. By exploring whether NIH neuron spike trains are compatible with a renewal process for their generation, we derived from their ISI pdfs expected auto-covariance functions under renewal assumptions (see Methods). By
comparison to these renewal (auto-covariance) functions, we found further evidence for intrinsic bursting tendencies in NIf\textsubscript{HVC} neurons. Of the 109 NIf\textsubscript{HVC} neurons tested, 57 exhibited auto-covariance functions that were smaller than the equivalent renewal functions somewhere in the time interval 3-45 ms (jackknife, p<0.01). Other than these cases, no significant differences with renewal statistics were observed. This absence of long sequences of short interspike intervals suggests a limitation of NIf\textsubscript{HVC} neurons to produce long bursts. In this respect, NIf\textsubscript{HVC} neurons are similar to HVC projection neurons, in which a similar trend was seen.

A different behavior was seen in NIf\textsubscript{I} neurons. Of the 13 NIf\textsubscript{I} neurons tested, 10 exhibited auto-covariance functions compatible with renewal assumptions, and 3 exhibited auto-covariance functions larger than renewal functions somewhere in the interval 3–31 ms. This behavior of NIf\textsubscript{I} neurons is reminiscent of RA and HVC\textsubscript{I} neurons, in which bursts were also found to be longer than expected by renewal statistics.

Given these diverse behaviors of individual NIf neurons, we tested their compatibility with renewal statistics also on the population level. We found that the median auto-covariance function of NIf\textsubscript{HVC} neurons was smaller than the median renewal function in the time interval 6–30 ms (Kruskal-Wallis test, p<0.01). However, the median auto-covariance function of NIf\textsubscript{I} neurons was not significantly different from the median renewal function (Kruskal-Wallis test, p<0.01, Fig. 3D). In conclusion, renewal tests in two NIf neuron types reveal differences between their burst generation mechanisms. Some form of depressive dynamics seems to shorten NIf\textsubscript{HVC} neuron bursts, whereas no such mechanism appears to apply to NIf\textsubscript{I} neurons.

NIf\textsubscript{HVC} neurons display a firing decrement in their autocorrelation function. In the range of about 10–100 ms, the average auto-correlation function of NIf\textsubscript{HVC} neurons clearly dips below its asymptotic value (Fig. 3E). In this time range, NIf\textsubscript{HVC} neurons exhibit a reduced spike probability down to 40% of baseline. We have not observed such a firing decrement in any other neuron type in NIf, HVC, RA, or LMAN; it may represent a soft refractory period unique to NIf\textsubscript{HVC} neurons.

Reversible lesions in NIf
To determine the role of NI₁HVC population activity for the generation of sleep bursts in HVC, we examined the effect of reversible inactivation of NI₁ while recording activity in single HVC₁ neurons (Fig. 4). When we injected the sodium-channel blocker lidocaine into NI₁, the spontaneous bursting of HVC₁ neurons immediately stopped (Fig. 4A) and recovered after about 3-4 minutes (Fig. 4A, bottom). The HVC₁ neuron burst rate in the 60s interval following the injection was significantly smaller than in the 60 second interval preceding the injection (Mann-Whitney U test, p<0.01, n=3/3 birds, 3 injections per bird, Fig 4C). Saline injections did not lead to a significant change in HVC₁ neuron burst rates (Mann-Whitney U test, p<0.01, n=3/3 birds, 3 injections per bird). We realized that a caveat of our lidocaine injections was that they might also have affected fibers of passage from Uva to HVC. To restrict the effect of injections to neural dendrites and somata in the NI₁ region, we also injected the GABAₐ agonist muscimol into NI₁. Similarly as for the lidocaine injections, we observed an immediate and significant reduction in HVC₁ neuron burst rate in the minute following the muscimol injection (Mann-Whitney U test, p<0.01, n=3/3 birds, 3 injections per bird, p<0.01, Fig. 4B). Recovery of burst rates was not observed within more than 20 minutes following muscimol injections. Despite these reduced burst rates, we found that HVC₁ neurons kept spiking after NI₁ inactivation (see Figures 4A and B), suggesting that lidocaine and muscimol did not leak into HVC. Furthermore, the average firing rate of HVC₁ neurons in the 50 seconds after lidocaine or muscimol injections into NI₁ was 10.2±5.4 Hz and so was smaller than the typical firing of HVC₁ neurons during sleep: 15±9.0 Hz (Hahnloser et al., 2006), but was not significantly different from average firing rates of these neurons measured in the awake bird: 7.2±7.0 Hz (Hahnloser et al., 2006). These results suggest that NI₁ provides essential input to HVC that is necessary to transform the regular spiking of HVC₁ neurons in the awake state into bursting in the sleeping state. The reduced firing and bursting of HVC₁ neurons after NI₁ inactivation agrees with previous inactivation and norepinephrine injection experiments in the anesthetized bird (Cardin and Schmidt, 2004b; Coleman and Mooney, 2004).

**Paired recordings in identified HVC and NI₁ neurons**
To directly compare firing properties of NIf neurons with those of HVC neurons, we performed paired recordings in these areas. NIf neuron type was identified by antidromic stimulation in HVC, and HVC neuron type was identified by antidromic stimulation in either RA or Area X.

From our single neuron recordings we know that NIf\textsubscript{HVC} neurons have a weak, but significant tendency to form burst epochs (in Figure 3C, in the time interval 0.75–3.4 seconds the median auto-covariance function is larger than the median renewal function, Kruskal-Wallis test, p<0.01). However, as to be expected, in paired recordings of NIf\textsubscript{HVC} and HVC\textsubscript{I} neurons, we observed much stronger burst epochs in HVC\textsubscript{I} neurons than in NIf\textsubscript{HVC} neurons (Fig. 5A). To analyze co-fluctuations of firing rates in more detail, we computed correlation coefficients of firing rates measured in non-overlapping 3 second windows. For the neuron pair shown in Figure 5A, the correlation coefficient of 0.15 was found to be significant (p < 0.05). In fact, most NIf\textsubscript{HVC}–HVC\textsubscript{I} neuron pairs showed significantly correlated firing rates (Fig. 5B, top left). By incorporating previously recorded data in HVC into our analysis (Hahnloser et al., 2006), we found that NIf\textsubscript{HVC}–HVC\textsubscript{I} firing-rate correlations are comparable to those of HVC\textsubscript{RA}–HVC\textsubscript{I} neurons (Fig. 5B, bottom left). Similarly, firing rates in most NIf\textsubscript{HVC}–HVC\textsubscript{RA} neuron pairs were significantly correlated, and also some NIf\textsubscript{HVC}–HVC\textsubscript{X} neuron pairs showed correlated firing rates. Thus, on the level of firing-rate correlations with HVC\textsubscript{I} neurons, there is a similarity between NIf and HVC projection neurons, despite their large differences in burst rates.

The firing-rate correlations involving interneurons in NIf and HVC are stronger and more reliable than those involving projection neurons in NIf and HVC. In 99 neuron pairs not involving a projection neuron in either HVC or NIF, we only found one pair that did not exhibit significantly correlated firing rates (Fig. 5B, right side). Average correlation coefficients for all neuron pairs studied are summarized in Table 2.

To further test for similarities between NIf\textsubscript{HVC} and HVC\textsubscript{RA} neurons, we compared firing rate distributions in paired recordings of NIf\textsubscript{HVC} and HVC\textsubscript{RA} neurons. Average firing rates in these neurons differed by about one order of magnitude (NIf\textsubscript{HVC}: 5.4±3.3 Hz, versus HVC\textsubscript{RA}: 0.6±0.9 Hz, n=34 pairs), as did average burst rates (NIf\textsubscript{HVC}: 1.4±0.9 Hz, versus HVC\textsubscript{RA}: 0.13±0.2 Hz, n=34 pairs). However, we found a striking similarity
between firing rate distributions of NIfhvc and HVCRa neurons after discounting for baseline differences in firing rates. That is, we counted the number of spikes fired in 100 ms windows of burst spike trains by only taking into account windows where at least one spike was found (thus discounting for differences in the number of empty windows). In 22 of 34 NIfhvc–HVCRa neuron pairs, we found indistinguishable firing rate distributions in the two cells (Kolmogorov-Smirnov test, p<0.01). Similarly, the distribution of firing rates of the entire NIfhvc population was identical to the distribution of the HVCRa population (Kolmogorov-Smirnov test, p<0.01, Fig. 5C). Equality of firing rate distribution in these different populations was robust and was observed for analysis windows in the range of the soft refractory time of NIfhvc neurons, i.e., from 50 ms to 150 ms. Firing similarity was less striking for other HVC neuron types. In 3 of 12 NIfhvc–HVCX neuron pairs we found equal firing rate distributions (also not counting empty windows). And, in only 3 of 37 NIfhvc–HVCI neuron pairs did we find equality of firing rate distributions (likewise not counting empty windows). Equality of firing rate distributions on the population level and in 100 ms windows was only observed for NIfhvc–HVCRa cell pairs.

To study the relationship between spikes in NIfhvc neurons and HVC projection neurons on a high temporal resolution, we examined raster plots of NIfhvc neuron activity aligned on HVCRa neuron bursts. We quantified the paired recordings by coherency functions, derived from the full spike trains including single spikes. We found that very often the HVCRa neuron bursts were preceded by NIfhvc neuron bursts by just a few milliseconds (Fig. 6A, B). For most NIfhvc–HVCRa neuron pairs, we found one significant peak in the coherency function (20/34 pairs exhibited each one significant peak at median time lag of −5 ms, in the range −21 to 1 ms; 14/34 pairs exhibited no significant peak, Fig 6C bottom). The average coherency function between NIfhvc and HVCRa neurons exhibited a peak at −5 ms, well above the average significance threshold (Fig. 6C, top). The median coherency function was above the median significance threshold in the time interval from −7 to −5 ms (Wilcoxon signed-rank test, p<0.01). Thus, NIfhvc neurons on average spike just a few milliseconds before HVCRa neurons do.
We also examined raster plots of NiFHVC activity aligned on HVCX neuron bursts. In many pairs, as was the case with HVCRA neurons, bursts in HVCX neurons tended to be preceded by NiFHVC neuron bursts. However, in some cases, bursts in the HVCX neuron preceded the NiFHVC neuron bursts (Fig. 6D). In summary, most NiFHVC–HVCX neuron pairs exhibited at least one significant coherency peak (8/12 pairs exhibited at least one significant peak at median time lag –2 ms, in the range –8 to 20 ms, Fig. 6E). The three main peaks at a positive time lag in Figure 6E are from three different birds and are unlikely to be a sampling artifact. The average coherency function between NiFHVC and HVCX neurons exhibited two distinct peaks, one peak at –3.5 ms, well above the average significance threshold, and the other at 15 ms, roughly coinciding with the average significance threshold (Fig. 6E, top). From these data, differences between the two types of HVC projection neurons are apparent. At a positive time lag larger than 5 ms there was no peak in 34 pairs with HVCRA neurons, whereas there were 6 peaks in 12 pairs with HVCX neurons. The only HVCRA neuron we found to somewhat reliably burst before the NiFHVC neuron was the one shown in Figure 6B. However, as can be seen, significance of the coherency function at a positive time lag was not achieved.

In paired recordings of NiFHVC neurons with HVCl neurons, we observed frequent bursts in the HVCl neuron in close proximity to NiFHVC neuron bursts (Fig. 7A). In most NiFHVC–HVCl neuron pairs there was at least one significant peak in the coherency function (32/37 pairs exhibited at least one significant peak with a median time lag of 7 ms, range –24 to 123 ms, Fig. 7B bottom). In agreement with the notion that NiFHVC neurons tend to burst before HVCl neurons, the average coherency function between NiFHVC and HVCl neurons exhibited a peak at 5 ms, well above the average significance threshold (Fig. 7B, top). The median coherency function of NiFHVC–HVCl pairs was significantly larger than the median significance threshold in the time interval from 2 to 14 ms (Wilcoxon signed-rank test, p<0.01).

We were not able to record from many NiFI neurons. An example raster plot of a NiFI neuron, simultaneously recorded with an HVCRA neuron is shown in Figure 7C. There was a highly significant coherency peak as a result of the many NiFI neuron bursts produced just a few milliseconds before the HVCRA neuron bursts. On the level of a small
population, we could verify this tendency (3/3 \( \text{HVC}_{\text{RA}} - \text{NIf}_1 \) pairs exhibited at least one significant peak with a median time lag of \(-9\) ms).

For the paired recordings of \( \text{NIf}_1 \) neurons with \( \text{HVC}_1 \) neurons, we did not produce raster plots due to the high burst densities in both of these neuron types. Coherency plots peaked significantly at a small negative time lag with respect to \( \text{HVC}_1 \) neuron spikes (3/3 significant pairs with median time lag of \(-4\) ms in the range \(-9\) to \(-2\) ms), showing that the \( \text{NIf}_1 \) neurons were strongly coherent with \( \text{HVC}_1 \) neurons (Fig 7D).

In conclusion, the time lags of coherency peaks were in agreement with a causal relationship between \( \text{NIf} \) neuron spikes and subsequent activity in \( \text{HVC} \). Some of our observations suggest interesting mechanisms by which \( \text{NIf} \) neurons drive bursts in \( \text{HVC} \) neurons during sleep, discussed in the following section.

**DISCUSSION**

We have found that \( \text{NIf} \) neurons can reliably be identified in extracellular recordings by antidromic stimulation from \( \text{HVC} \). Antidromic response latencies in \( \text{NIf}_{\text{HVC}} \) neurons were very short and narrowly clustered, suggesting that synchronized bursts in \( \text{NIf} \) could lead to short latency bursts in \( \text{HVC} \) with very little temporal jitter. Compared to \( \text{NIf}_{\text{HVC}} \) neurons, \( \text{NIf}_1 \) neurons responded to \( \text{HVC} \) simulation with much larger latency variability. We observed many distinguishing characteristics of spontaneous activity in these two different \( \text{NIf} \) neuron types, including mean firing rates, burst rates, and burst generation mechanisms.

Given the absence of any known projection target of \( \text{NIf} \) other than \( \text{HVC} \), we speculate that the second \( \text{NIf} \) neuron type belongs to a class of interneurons. One reason for our speculation are the high burst rates in \( \text{NIf}_1 \) neurons, analogous to interneurons in RA and HVC (Leonardo and Fee, 2005; Hahnloser et al., 2006). With RA- and HVC interneurons being inhibitory (Spiro et al., 1999; Rosen and Mooney, 2003), it may well be that \( \text{NIf}_1 \) neurons are inhibitory as well. This classification is quite plausible, given that our GABA and muscimol injection experiments and previous such experiments (Cardin and Schmidt, 2004a; Coleman and Mooney, 2004) strongly suggest an abundance of GABA receptors in \( \text{NIf} \). However, further experiments will be necessary to determine the neurotransmitter type released by \( \text{NIf}_1 \) neurons.
We suspect the high coherencies between NIf₁ and HVC neurons arises from common input from NIf₇HVC neurons, just as HVC₁ neurons are strongly coherent with RA neurons, likely by virtue of common input from HVC₉RA neurons (Hahnloser et al., 2006). Currently, the only evidence that NIf₁ neurons are synaptically driven by NIf₇HVC neurons is that they respond with short latency to HVC stimulation, shown here to antidromically activate NIf₇HVC neurons. More paired NIf₁ and HVC recordings would help to further clarify the relationship between NIf₁ and HVC activity.

We did not find significant differences between firing rates of NIf neurons in the awake and the sleeping states. This finding contrasts with a previous study where arousal of lightly sedated birds lead to reduction of spontaneous NIf activity (Cardin and Schmidt, 2004a). This apparent disagreement might arise from differences between the sedated state and the sleeping state or might be attributed to effects of residual melatonin in our experiments. In any case, we believe the most interesting phenomenon to be that NIf cells burst in the awake state, a property that has never been observed in either RA or HVC cells.

We speculate that there exists a mechanism that gates the response of HVC to ongoing drive from NIf, much as there is a mechanism that gates the transmission of auditory responses in HVC from NIf (Cardin and Schmidt, 2003; Cardin and Schmidt, 2004b). According to our view, NIf₇HVC neurons would constantly bombard and occasionally activate HVC projection neurons. Not all of these events would necessarily result in a dense sleep-burst epoch in HVC, to agree with the moderate firing-rate correlations of NIf₇HVC and HVC projection neurons with HVC₁ neurons in Figure 5. However, on some occasions, a dense sleep-burst epoch would form, activating most HVC neurons. Since we found a weak but non-negligible tendency of NIf₇HVC neurons to also form sleep-burst epochs (Figs. 3C, 5A, B), we speculate that Uva might somehow control the generation of sleep-burst epochs, for example, by coordinating neuromodulatory input (Akutagawa and Konishi, 2005). Further influences on HVC excitability might also result from input from the medial magnocellular nucleus of the anterior nidopallium (Foster et al., 1997; Foster and Bottjer, 2001), as well as from direct cholinergic (Shea and Margoliash, 2003) or other neuromodulatory input to HVC (Appeltants et al., 2000).
To our surprise, despite large differences between average firing rates in NIf- and HVC projection neurons, we have also found many similarities on a global and a local level. First, NIf\textsubscript{HVC} and HVC\textsubscript{RA} neurons have a similar burst density on the level of the entire population, when considering that burst rates in NIf\textsubscript{HVC} neurons were ten times higher, but their population size is about ten times smaller. Also, thanks to their soft refractoriness on the time scale of song syllables (~100 ms), firing-rate distributions of NIf\textsubscript{HVC} neurons were found to be identical to those of HVC\textsubscript{RA} neurons (Fig. 5C).

A central question pertaining to NIf output is whether NIf activity serves only to initiate a sequence of self-sustained sleep bursts in HVC or whether every single burst in HVC is driven by a particular set of NIf bursts. In the former case, a particular set of strongly synchronized bursts in NIf might serve as the single initialization signal for the HVC bursts. In the latter case, transiently increased burst rates in NIf might lead to repeated excitation of HVC neurons beyond their firing thresholds. Our paired recordings involving NIf\textsubscript{HVC} neurons can be helpful to tease these two possibilities apart. Our main observation was an interesting difference in the coherencies of NIf\textsubscript{HVC} neurons with the two types of HVC projection neurons. HVC\textsubscript{RA} neurons burst reliably just after NIf\textsubscript{HVC} neurons did, whereas HVC\textsubscript{X} neurons burst reliably after \textit{and} before NIf\textsubscript{HVC} neurons. From the coherencies with HVC\textsubscript{RA} neurons we would be led to conclude our first hypothesis — that NIf activity serves to initialize HVC sleep-burst sequences — is correct. However, the HVC\textsubscript{X} bursts preceding the NIf\textsubscript{HVC} bursts suggest that, at least on the timescale of a few milliseconds, NIf neurons engage in stereotyped sequential activity. Thus, the more complete picture suggested by our data is that there is a first population burst in NIf that activates mainly HVC\textsubscript{X} neurons. Then, a second population burst just a few milliseconds later activates HVC\textsubscript{RA} and HVC\textsubscript{X} neurons. Such a scenario can explain why HVC\textsubscript{RA} bursts are rarely observed to precede NIf\textsubscript{HVC} bursts, whereas HVC\textsubscript{X} neurons are observed to frequently burst before and after NIf\textsubscript{HVC} neurons. It is interesting to note that, in our previous recordings of HVC neuron pairs (Hahnloser et al., 2006), while most HVC\textsubscript{X} - HVC\textsubscript{RA} pairs were not coherent, the single pair we found that showed coherent bursting did so with the HVC\textsubscript{X} neuron leading by roughly 20 ms. Coherency differences between the two types of HVC projection neurons might result from two distinct classes of NIf\textsubscript{HVC} neurons, from HVC-intrinsic mechanisms such as inhibition (Mooney, 2000; Solis and
Perkel, 2005), or from other brain areas such as Uva. For example, Uva projection neurons might directly drive HVC$_X$ neurons and indirectly drive HVC$_{RA}$ neurons via activation of NIf.

We can speculate about the functional role of the observed latency differences between HVC$_{RA}$ and HVC$_X$ neurons. It is clear that signals originating in HVC and arriving in RA take longer if they do so by traveling along the anterior forebrain pathway involving Area X (Okuhata and Saito, 1987) than by traveling directly from HVC to RA. Thus, the anticipatory firing of HVC$_X$ neurons can partially compensate for signal delays that would normally occur if they fired simultaneously with HVC$_{RA}$ neurons. The signal propagation time from HVC to RA along the anterior forebrain pathway has been estimated to be around 40 ms (Troyer and Doupe, 2000; Kimpo et al., 2003; Abarbanel et al., 2004). With our measured time difference of 20 ms (15+5 ms) between the second peak in HVC$_X$ coherency functions and the first peak in HVC$_{RA}$ coherency functions, it follows that about half the signal propagation time along the anterior forebrain pathway is compensated for by anticipated departure of HVC$_X$ neuron signals. With an estimated propagation time of 4 ms for HVC$_{RA}$ signals to reach RA (Hahnloser et al., 2006), we conclude that bursts in NIf tend to reach RA via the premotor pathway 16 ms before they reach RA via the anterior forebrain pathway.

What could be the purpose of such a latency adjustment? Some previous models of vocal learning have emphasized the importance of differences in propagation delay through the premotor pathway and through the anterior forebrain pathway. In these models HVC$_X$ activity is to be viewed as an efference copy of premotor signals in HVC$_{RA}$ neurons (Troyer and Doupe, 2000; Abarbanel et al., 2004). Given the known modulatory role of LMAN for song (Scharff and Nottebohm, 1991; Kao et al., 2005; Olveczky et al., 2005), anticipation of HVC signals transmitted along the anterior forebrain pathway could be important to maintain proper registration of premotor signals along the two efferent pathways of HVC.

Given our acute experimental protocol, we were unable to relate individual spike patterns in the sleeping bird to premotor activity during singing. Therefore, we analyzed burst events, knowing that bursting in HVC and in RA is a signature of the singing state. Our reversible inactivation experiments provide new insights into the role of NIf for burst
generation in HVC (Figure 4). Given the strong correlations between HVC₁ neurons and other HVC neuron types during sleep (Hahnloser et al., 2006), and given the strong reduction of auditory-evoked subthreshold activity in all HVC neuron types after NIf inactivation (Coleman and Mooney, 2004), we speculate that NIf drives sleep bursts in all HVC neuron types, and presumably also in RA. Such a role of NIf during sleep is highly remarkable given that NIf neurons exhibit singing-related activity (McCasland, 1987; Hahnloser and Fee, 2003), but HVC seems to be able to produce song-related bursts in adult zebra finches with bilateral NIf lesions (Cardin et al., 2005). In combination with the loss of auditory responses in HVC neurons after NIf inactivation (Coleman and Mooney, 2004), our results imply a surprising involvement of NIf in conveying song-specific activity, both on a replay and on a sensory level. With the possibility of existence of offline learning mechanisms during sleep (Nadasdy et al., 1999; Margoliash, 2001; Deregnaucourt et al., 2005), we could imagine that auditory hallucination of the BOS originating in NIf is the driving force of such memory consolidation processes.

FIGURE CAPTIONS

Figure 1 NIf and NIf neuron identification. A) Schematics of the song-control system and the experimental setup. Nucleus interface of the nidopallium (NIf) receives input from the thalamic nucleus uveaformis (Uva) and projects to HVC. HVC projects to the robust nucleus of the arcopallium (RA), which in turn innervates vocal motor neurons in the hypoglossal nucleus (nXIIIts). NIf neuron type was identified by antidromic stimulation in HVC, and HVC neuron type by antidromic stimulation in RA or Area X. B) NIf extent, as assessed by the region of increased response to stimulation agrees with histological identification. NIf has been retrogradely labeled by BDA injection in HVC. The two white circles close to the extremes of the white line mark electrolytic lesions used as reference points along the electrode track. Shown in the tilted axes is the root-mean square (RMS) extracellular voltage amplitude in the interval 1–7 ms following stimulation onset. The RMS amplitude is high (>~1 mV) when the electrode is inside NIf.
and low otherwise. C) Electrical stimulation in HVC elicits spike responses in \text{NIHVC} neurons at a single precise time lag (top). Stimulating 0.5 ms after a spontaneous spike in a \text{NIHVC} neuron leads to response failure (bottom). D) Antidromic responses in \text{NI1} neurons have larger latency variability (top) and do not fail when HVC is stimulated right after a spontaneous spike (bottom). E) A plot of spike latency versus latency variability allows for segregation of \text{NIHVC} neurons from \text{NI1} neurons based on a latency-variability threshold of 0.1 ms. F) Spike waveforms of \text{NIHVC} neurons (top) and of \text{NI1} neurons (bottom) are very similar.

**Figure 2** \text{NI} neuron spike trains during sleep. A) Extracellular records of a \text{NIHVC} neuron (top) and a \text{NI1} neuron (bottom). The burst events labeled on the left are redrawn on a smaller time scale on the right. B) Instantaneous firing rate (IFR) functions of a \text{NIHVC} neuron (top) and a \text{NI1} neuron (bottom). C) Firing rate distribution of \text{NIHVC} neurons and D) \text{NI1} neurons in three second windows. Filled symbols are average firing rates in regular spike trains, and open symbols are from burst spike trains with single spikes removed. The numbers shown on the right are average firing rates plus/minus standard deviations for the burst spike trains, computed after removing single spikes.

**Figure 3** Interspike interval (ISI) probability density functions (pdfs) and autocorrelation functions. A) Average ISI pdf of \text{NIHVC} neurons, with the shaded area representing plus/minus the standard deviation. There are few ISIs of ~10 ms and few ISIs larger than one second. B) The average ISI pdf of \text{NI1} neurons shows virtually no ISIs larger than 400 ms. C) Average auto-covariance function of \text{NIHVC} neurons plotted on a logarithmic time scale. The delta-function peak at time zero has been suppressed. The dashed line is the average renewal auto-covariance function derived from the respective ISI pdfs. In the interval close to 10 ms, the auto-covariance function is smaller than the renewal function, and in the interval around 1 second, it is larger (as indicated by the two thick horizontal bars below the auto-covariance function). The inset shows the auto-covariance function of the \text{NIHVC} neuron from Figure 2B, reflecting stereotyped bursting behavior by the oscillatory shape. D) The average auto-covariance function of \text{NI1} neurons is larger than
the renewal function, but this difference is not significant. The inset depicts the auto-
covariance function of the NIf neuron from Figure 2B. E) Average autocorrelation
function of NIfHVC neurons plotted on a linear time scale, clearly displaying the soft
refractory period of about 125 ms during which the spiking probability is reduced down
to at most 40 percent of baseline.

Figure 4 Effect of lidocaine and muscimol injections into NIf on sleep burst rate in HVC neurons. The numbers separated by a backlash indicate the number of bursts measured in
the 50 second interval before the injection and after the injection. A) Lidocaine injections
into NIf immediately disrupt bursting in HVC neurons as can be seen from the
instantaneous firing rates of two HVC neurons (top and bottom). Bursting recovers
within 3-4 minutes (bottom). B) Muscimol injections into NIf have a similar effect of
reducing burst rate in HVC neurons, but no recovery is observed on this timescale. C)
Median post-injection burst rates of HVC neurons were significantly lower than median
pre-injection rates for both muscimol and lidocaine injections. Burst rates recovered after
5 minutes of lidocaine injections to values similar to pre-injection rates. The error bars
delimit the upper and lower quartiles and the asterisks denote significant differences
(Mann-Whitney U test, p<0.01).

Figure 5 Burst density variations in NIfHVC and HVC neurons are weakly correlated. A)
Instantaneous firing rates in a simultaneously recorded NIfHVC–HVC neuron pair. The
clustering of HVC neuron spikes into burst epochs is only weakly reflected in the NIfHVC
neuron (two burst epochs in the HVC neuron that seem to be coincident with increased
bursting in the NIfHVC neuron are marked by shaded areas). B) Correlation coefficients
of firing rates measured in 3 second windows of simultaneously recorded neuron pairs.
Black bars represent significantly correlated pairs (p<0.01). Correlations among neuron
pairs involving HVC or NIf projection neurons are often not significant and smaller (left
row) than correlations among other neuron pairs (right row). The arrow indicates the
correlation coefficient of the neuron pair in A. C) By performing the same analysis as in
Figure 2C, but on a time window of 100 ms instead of 3 seconds, we find identical firing-
rate distributions in NIfHVC and HVCRA neurons. To produce this subfigure we analyzed
burst-train data from 34 simultaneously recorded neuron pairs. As illustration, the ‘probabilities’ of zero spikes in NIffHVC and HVCRA neurons are also shown; these differ strongly, but are not taken into account for comparing the distributions.

**Figure 6** Paired recordings of NIff neurons and HVC projection neurons. A) Spike raster plot of simultaneously recorded NIffHVC and HVCRA neuron pair. The spike bursts of the HVCRA neuron (short red lines) are aligned on the first spike at the center of the plot. Corresponding NIffHVC spikes (short black lines) are shown below each HVCRA burst. NIffHVC neuron bursts tend to precede the HVCRA neuron bursts by a few milliseconds. Below the raster plot is the coherency function plotted as a black line, with the significance threshold shown by the dashed line. The full line exceeds the dashed line in a peak at ~8 ms, indicating significant coherency. B) Same analysis for another NIffHVC–HVCRA neuron pair. Again, significant coherency exists at a negative time lag. The few bursts in the NIffHVC neuron that follow the HVCRA neuron bursts are not significant as can be seen in the bottom. C) Summary of all HVCRA–NIffHVC neuron pairs. Top: The full line shows the average coherency function between all HVCRA–NIffHVC neuron pairs. The shaded area delimits the standard deviation of coherency functions. The dashed line shows the average significance threshold. There is a narrow coherency peak at a negative time lag where the median coherency is larger than the median significance threshold (thick horizontal bar). Bottom: All significant coherency peaks from individual neuron pairs. D) Raster plot of a HVCX–NIffHVC neuron pair. The NIffHVC neuron bursts reliably after the HVCX neuron (with significant coherency). E) Summary of HVCX–NIffHVC neuron pairs. The average coherency function (top) exhibits significant peaks at ~3.5 and at 15 ms. Peaks of individual coherency functions are found at both positive and negative time lags in the range ~40 to 100 ms. Full circles denote the main peaks of individual neuron pairs and open circles denote significant secondary peaks (of smaller coherency value than the main peak).

**Figure 7** Raster plots involving HVC1 and NIff1 neurons. A) Raster plot of HVC1 neuron spikes, time-aligned on NIffHVC neuron bursts. Below, the coherency function peaks 1 ms
after NIf\textsubscript{HVC} neuron spikes. B) The average coherency function of NIf\textsubscript{HVC}–HVC\textsubscript{I} neurons peaks 5 ms after NIf\textsubscript{HVC} neuron spikes (top). Individual peaks are almost exclusively located at positive time lags of NIf\textsubscript{HVC} neuron spikes. C) Raster plot of NIf\textsubscript{I} neuron spikes, time-aligned to HVC\textsubscript{RA} neuron bursts. The coherency function significantly peaks –9 ms before HVC\textsubscript{RA} neuron spikes (bottom). D) The average coherency function of HVC\textsubscript{I}–NIf\textsubscript{I} neuron pairs peaks –6 ms before HVC\textsubscript{I} neuron spikes (top). All three neuron pairs exhibit significant coherency peaks at a small negative time lag (bottom).

### TABLES

<table>
<thead>
<tr>
<th>Neuron type</th>
<th>Firing rate (s(^{-1}))</th>
<th>Burst firing rate (s(^{-1}))</th>
<th>Burst rate (s(^{-1}))</th>
<th>Burst width (ms)</th>
<th>Spikes per burst</th>
<th>Spikes in bursts (%)</th>
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<td>NIf\textsubscript{HVC} (n=117)</td>
<td>5.4±3.3</td>
<td>461±139</td>
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<td>550±173</td>
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<td>3.7±1.2</td>
<td>2.5±0.3</td>
<td>58±19</td>
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<td>NIf\textsubscript{I} (n=13)</td>
<td>27.8±9.6</td>
<td>321±87</td>
<td>4.9±2.9</td>
<td>7.5±1.7</td>
<td>3.0±0.9</td>
<td>53±24</td>
</tr>
</tbody>
</table>

**TABLE 1**: Firing statistics for two NIf neuron types. All values are means plus/minus standard deviation.

<table>
<thead>
<tr>
<th>Neuron pair</th>
<th># correlated pairs</th>
<th>Corr. Coeff.</th>
<th>Med. burst ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIf\textsubscript{HVC}–HVC\textsubscript{I}</td>
<td>22/37, 13 birds</td>
<td>0.42±0.17</td>
<td>1.2</td>
</tr>
<tr>
<td>HVC\textsubscript{I}–NIf\textsubscript{I}</td>
<td>3/3, 3 birds</td>
<td>0.51±0.15</td>
<td>3.2</td>
</tr>
<tr>
<td>HVC\textsubscript{RA}–NIf\textsubscript{HVC}</td>
<td>19/33, 12 birds</td>
<td>0.28±0.11</td>
<td>19.6</td>
</tr>
<tr>
<td>HVC\textsubscript{RA}–HVC\textsubscript{I}</td>
<td>16/26, 6 birds</td>
<td>0.42±0.12</td>
<td>46.8</td>
</tr>
<tr>
<td>HVC\textsubscript{X}–NIf\textsubscript{HVC}</td>
<td>2/12, 4 birds</td>
<td>0.29±0.01</td>
<td>-</td>
</tr>
<tr>
<td>HVC\textsubscript{I}–HVC\textsubscript{I}</td>
<td>19/19, 5 birds</td>
<td>0.91±0.06</td>
<td>-</td>
</tr>
<tr>
<td>RA–RA</td>
<td>28/29, 3 birds</td>
<td>0.79±0.1</td>
<td>-</td>
</tr>
<tr>
<td>RA–HVC\textsubscript{I}</td>
<td>50/50, 9 birds</td>
<td>0.66±0.19</td>
<td>3.4</td>
</tr>
</tbody>
</table>
TABLE 2: Sleep-related firing-rate correlations and burst ratios in different neuron pairs, measured in non-overlapping 3 second windows. The criterion for significance was $p < 0.05$. Correlation coefficients (mean plus/minus standard deviation) were computed for significantly correlated pairs only. The median burst-rate ratios in the last column were computed by the median value of ratios of average firing rates in simultaneously recorded pairs of neurons, single spikes removed. In the denominator for this ratio, we always choose the neuron appearing on the left in the first column. For example, NIf neurons fired 3.2 times as many spikes in burst trains as did HVC neurons.

REFERENCES


Figure 1
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7