ARTICLES

Using temperature to analyse temporal dynamics in the songbird motor pathway

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Many complex behaviours, like speech or music, have a hierarchical organization with structure on many timescales, but it is not known how the brain controls the timing of behavioural sequences, or whether different circuits control different timescales of the behaviour. Here we address these issues by using temperature to manipulate the biophysical dynamics in different regions of the songbird forebrain involved in song production. We find that cooling the premotor nucleus HVC (formerly known as the high vocal centre) slows song speed across all timescales by up to 45 per cent but only slightly alters the acoustic structure, whereas cooling the downstream motor nucleus RA (robust nucleus of the arcopallium) has no observable effect on song timing. Our observations suggest that dynamics within HVC are involved in the control of song timing, perhaps through a chain-like organization. Local manipulation of brain temperature should be broadly applicable to the identification of neural circuitry that controls the timing of behavioural sequences and, more generally, to the study of the origin and role of oscillatory and other forms of brain dynamics in neural systems.

Motor behaviours are built out of a sequence of movements that evolve through time. From the most basic, such as locomotion, to the most complex, such as playing the piano, the timing of movements is crucial. For some simple oscillatory behaviours, in which the movement evolves on a single timescale, it has been possible to identify the particular neurons and biophysics that control the temporal dynamics of the behaviour—for example pacemaker neurons in the stomatogastric ganglion[1] or the oscillator network that controls swimming in the leech[2]. However, it is not known what mechanisms underlie more complex learned behaviours that have structure on many timescales.

Birdsong has a remarkably precise and hierarchically organized temporal structure[3,4] mediated by a number of distinct, well-studied motor nuclei[5,6] (Fig. 1a), allowing for an unprecedented view into the central control of motor timing. Adult zebra finches generate a 0.5–1.0-s song motif that is repeated a number of times during a bout of singing[7]. The motif itself is made up of song syllables—individual bursts of sound that are approximately 100 ms in length and occur in a precise order. Syllables are highly stereotyped and often contain complex acoustic structure that can evolve rapidly (10-ms timescale). The duration of song elements at all timescales is stereotyped; trial-to-trial fractional variations in song timing are roughly 1% (refs 8–10)z.

It is not known whether different brain regions are responsible for the timing of motifs, syllables, and subsyllabic structure. Two forebrain nuclei in particular have been implicated in the control of the temporal structure of song: HVC and RA. HVC projects to RA, which in turn projects to the vocal motor neurons[11] as well as midbrain vocal control and brainstem respiratory areas[12]. Previous electrophysiological studies have found evidence that these brain regions contribute to song structure in a hierarchical manner[13,14] and have suggested that the dynamics underlying the generation of different song timescales may reside in different brain regions. For instance, syllable-timescale dynamics have been suggested to occur in HVC[15], whereas subsyllable-timescale dynamics may arise in RA[14,16,17]. Additionally, portions of the midbrain and respiratory areas project back to HVC through thalamic nucleus Uvaeformis (Uva)[18,19], raising the further possibility that syllables, which are tightly linked to respiratory patterning[20], may be timed by respiratory oscillator circuits[9,21]. With current techniques, however, it has been difficult to test ideas about the origin of dynamics that underlie the temporal control of song.

Localizing temporal dynamics with temperature

We set out to localize temporal dynamics within the song control system, taking advantage of the fact that the speed of brain processes is strongly temperature dependent[22–24]. The aim was to produce localized mild heating or cooling[25,26], rather than inactivation, for which cooling has also been used[27]. The basic logic of our experiments is as follows. If the circuitry in a particular brain area is involved in controlling song timing, then cooling that area should slow the song. Furthermore, if the neural control of song is organized with a dynamical hierarchy (that is, different song timescales are controlled by biophysical dynamics in different brain areas), it should be possible to differentially alter the behavioural timescales by individually manipulating the temperature in these areas.

Dynamics in HVC

We started by bilaterally manipulating the temperature of nucleus HVC. We designed a device, based on the Peltier effect, that is capable of rapidly heating or cooling HVC in a spatially restricted manner (Fig. 1a–c, Supplementary Fig. 1). Song timing was strongly affected by changes in HVC temperature. At colder temperatures, song motifs were produced more slowly than control songs (Fig. 1d, Supplementary Fig. 2). All birds ($n = 10$) showed a significant increase in motif duration during cooling (ranging from 16.9 to 44.9%; Fig. 1e). Fractional change in motif duration (dilation) was found to vary approximately linearly with temperature in the range from 0 to $-6.5\ ^{\circ}\mathrm{C}$ (0 to 1 A in terms of current; Fig. 1f). The slope of this relation was used as a simple metric of temperature-dependent song dilation, which we refer to as stretch (measured in per cent per degree Celsius; see Supplementary Methods). The stretch metric in different birds ranged from -1.89 to $-3.97\%\ ^{\circ}\mathrm{C}^{-1}$, with a mean of $-2.83 \pm 0.22\%\ ^{\circ}\mathrm{C}^{-1}$. Changes in song speed during cooling were immediate and persisted for an hour or more (Supplementary Fig. 1d). Notably, temperature changes in HVC had only a small effect on the acoustic structure of the song (Supplementary Fig. 4).

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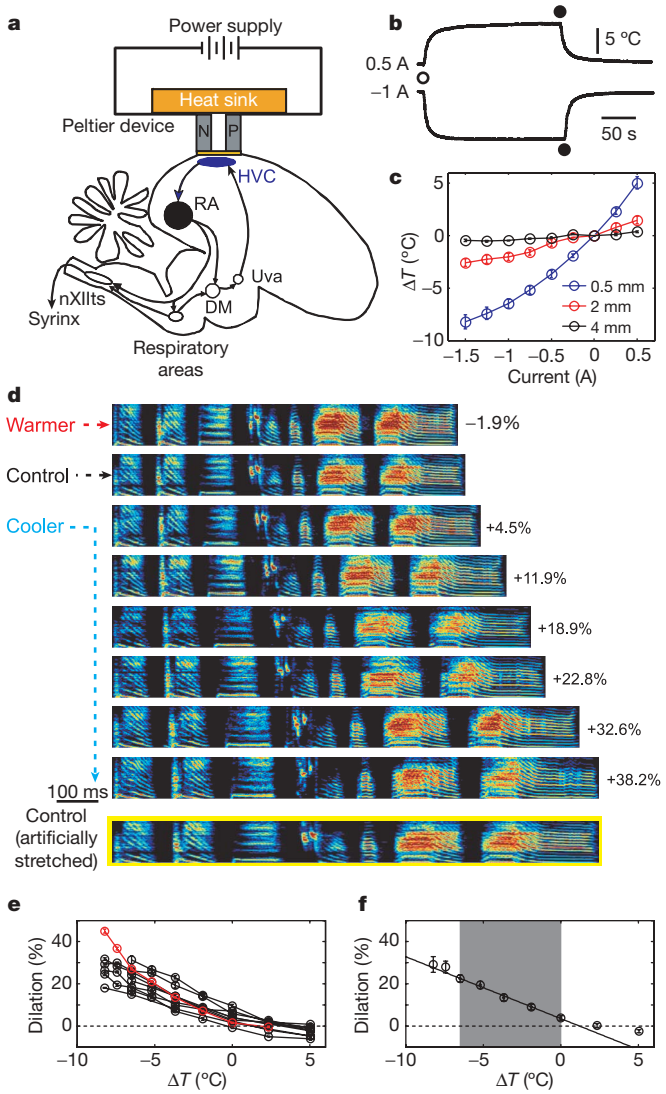

Figure 1 | Changes in HVC temperature affect song duration. **a**, The Peltier device and relevant parts of the song production pathway. N and P, semiconductor elements; DM, dorsomedial nucleus of the intercollicular complex; nXIIts, tracheosyringeal part of the hypoglossal nucleus. **b**, Temperature change in HVC as a function of time after onset (open circle) of the indicated current through the Peltier device (top, heating; bottom, cooling); current switched off at filled circles. **c**, Calibration curves for brain temperature changes (ΔT) at various depths under the Peltier device ($n = 4$). **d**, Representative sonograms (frequency, 1–9 kHz) recorded from bird no. 3 with HVC heated (0.25 A) and cooled (0.25 to 1.5 A in 0.25-A steps), showing percentage song dilation relative to control. Hotter colours represent greater sound intensity. Bottom, spectrogram of the control motif shown artificially stretched. **e**, Percentage change in duration (dilation) of song motif versus change in temperature, relative to the pre-implantation song ($n = 10$). Red, bird no. 3. **f**, Motif dilation averaged over all ten birds. The shaded area represents the range over which the song stretch metric (slope) was calculated. All error bars, s.e.m.

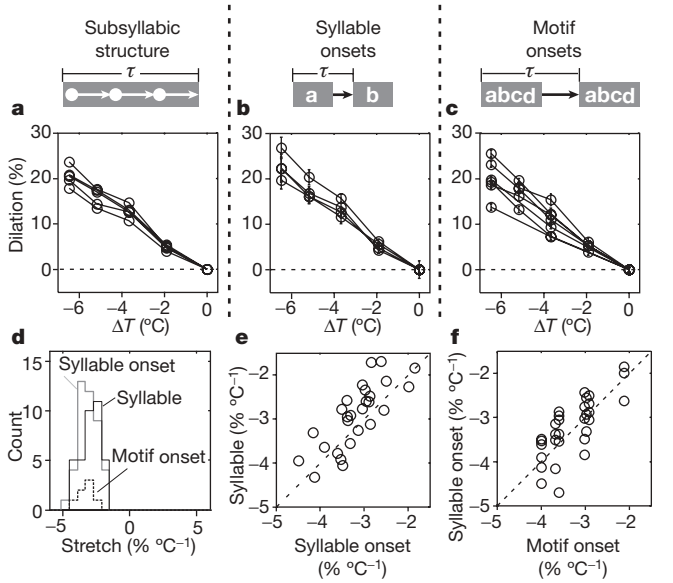

Figure 2 | HVC cooling slows the song at all timescales. **a**, Dilation of subsyllabic structure versus HVC temperature change for all five syllables of bird no. 8. **b**, Dilation of syllable-onset intervals for the bird no. 8. **c**, Dilation of motif-onset intervals for all seven birds that produced concatenated motifs at all temperatures. All error bars, s.e.m. **d**, Distribution of stretch metrics for the entire data set, including syllables (36 syllables, eight birds), syllable onsets (43 syllables, nine birds) and motif onsets (seven birds). **e**, **f**, Stretch of syllable-onset interval was strongly correlated with subsyllabic stretch (**e**) and motif-onset stretch (**f**) (for further details, see Supplementary Information).

To quantify whether HVC cooling slows the song even at the shortest timescale of subsyllabic structure, we used a standard dynamic time warping algorithm based on the correlation of sound features of the control song with the cooled song (Supplementary Methods, Supplementary Fig. 5), and also directly measured the duration of subsyllabic elements. The average dilation of subsyllabic structure for each song syllable was computed at each temperature condition (0 to −6.5 °C; Fig. 2a), and the slope (the stretch metric) of the dilation as a function of temperature was computed for each syllable (Fig. 2d). The mean stretch for subsyllabic structure was found to be $-2.88 \pm 0.12\%\ ^{\circ}C^{-1}$, which differs significantly from zero (*t*-test, $P < 10^{-6}$). This observation suggests that biophysical dynamics in HVC are involved in controlling song timing on a fine timescale.

We then considered the control of syllable onsets. In principle, respiratory circuits projecting to HVC (for example through Uva) could act as a 'clock' that autonomously controls the initiation of syllables, in which case cooling HVC should have little effect on syllable onsets. Alternatively, the onset of a syllable may be linked to the completion of the previous syllable, in which case the interval between onsets should increase during cooling, as the duration of each syllable increases. In fact, the intervals between syllable onsets were significantly dilated by an average of $-3.05 \pm 0.11\%\ ^{\circ}C^{-1}$ (*t*-test, $P < 10^{-6}$; Fig. 2b, d). This is not consistent with a model in which syllable timing (or the timing of singing-related respiration) is autonomously controlled by circuit dynamics in respiratory circuits or any other area upstream or downstream of HVC.

The stretch of syllable-onset intervals was significantly correlated with the stretch of the syllables within the intervals ($r^2 = 0.607$, slope of 0.92 ± 0.16; Fig. 2e), and more weakly correlated with the stretch of other syllables ($r^2 = 0.47$; see Supplementary Materials). In other words, for syllables that had a larger stretch than average, the onset interval to the following syllable also had a larger stretch than average. This is consistent with a model in which the onset of each syllable may be causally linked to, or triggered by, the end of the previous syllable.

The silent gaps between syllables were also significantly dilated by HVC cooling ($-3.70 \pm 0.32\%\ ^{\circ}C^{-1}$; *t*-test, $P < 10^{-12}$, median of $-3.29\%\ ^{\circ}C^{-1}$), suggesting that biophysical dynamics in HVC are involved in the timing of gaps. The cooling-related stretch of gaps was slightly larger than that observed for syllables (paired *t*-test, $P < 0.05$, median gap stretch was 12% larger than median syllable stretch). Similarly, the average stretch of a syllable-onset interval was slightly larger than the stretch of the syllable contained within that interval (mean paired difference, $0.26 \pm 0.088\%\ ^{\circ}C^{-1}$; paired *t*-test, $P < 0.001$, median interval stretch was 3.4% larger than median syllable stretch; Supplementary Fig. 6). These observations imply that the circuit mechanisms involved in initiating song syllables may be different from those involved in generating structure within song

syllables, as has been suggested by measurements of the variability in timing of gaps and syllables in natural singing[8,9].

An analogous argument can be made for the timing of motif onsets; if there is a 'motif clock' outside HVC that independently controls the intervals between motif onsets, then cooling HVC should have little effect on motif-onset intervals. In fact, motif-onset intervals were significantly dilated by an average of $-3.19 \pm 0.24\%\ ^{\circ}C^{-1}$ (*t*-test, $n = 7$, $P < 10^{-5}$; Fig. 2c, d, f), which is not consistent with a model in which motif onsets are timed autonomously by circuit dynamics outside HVC.

Dynamics in RA

Although the HVC cooling experiments strongly suggest the involvement of HVC in generating the fine temporal structure within syllables, they do not rule out some involvement of other brain areas. In particular, circuit dynamics[14,16,17] and connectivity[28,29] within RA, as well as reciprocal connections from RA to HVC[30], have been implicated in the generation of these short timescales. In general, these models would predict that song timing can be affected by manipulating circuit dynamics in RA. Here we directly test this prediction by bilaterally cooling RA during singing. We use a Peltier device similar to that used for HVC, but with attached gold probes (330-μm diameter) that were implanted into RA to facilitate thermal conduction (Fig. 3a, Supplementary Figs 7, 8). At a distance of 200 μm from the probe, the distance estimated to be the farthest extent of RA neurons, we observed a temperature drop of 10 °C at the maximum current used. We also found that the RA cooling device produced a slight temperature change in HVC of roughly 30% of the temperature change in RA at each current level (Fig. 3b).

As expected, because of the residual effect of the RA cooling probe on HVC temperature, we observed a slight increase in motif duration at higher cooling currents ($n = 4$; Fig. 3c, Supplementary Fig. 9a). The effect of the residual HVC cooling on motif duration can be accurately predicted by the results of the HVC cooling experiments (Fig. 1f) and fully accounts for changes in motif duration produced by the RA probe (Fig. 3d). Thus, after incorporating a correction for HVC temperature changes, we find no evidence that changes in RA temperature affect song motif duration ($P > 0.20$; Fig. 3e) or the timing of subsyllabic structure (Fig. 3f), suggesting that dynamics in RA may not contribute significantly to song timing, at least by any mechanism that is sensitive to temperature changes in the range we were able to achieve here.

The fact that RA cooling had so little effect on song structure led us to wonder whether our temperature manipulation had any effect on the neuronal properties in RA. In non-singing birds, RA neurons spontaneously generate tonic, regular spiking[14], possibly associated with an intrinsic subthreshold membrane potential oscillation[31]. We measured the spiking frequency of single units in RA in an anaesthetized preparation while changing the temperature using the RA cooling device. Cooling produced a rapid, roughly linear decrease in RA neuron tonic spiking rate (19 cells from seven birds, slope of $0.85\ Hz\ ^{\circ}C^{-1}$; Fig. 4a–c and Supplementary Fig. 9b) that resulted in a near cessation of spontaneous spiking at the coldest temperatures ($\Delta T = -10\ ^{\circ}C$). Our observation that cooling RA by 10 °C produces a 2.5-fold reduction in the intrinsic oscillation frequency of RA neurons, yet has no detectable effect on song structure or timing, implies that these oscillations are not likely to be a source of dynamics underlying song production.

In contrast, the incidence of high-frequency spontaneous bursts in RA (Fig. 4d, top), known to be driven by synaptic input from HVC under anaesthesia and during sleep[32,33], does not show a significant trend with temperature ($P > 0.6$; Fig. 4d). The bursts exhibited only a slight cooling-related decrease in firing rate ($5.6\ Hz\ ^{\circ}C^{-1}$; Supplementary Fig. 9c). RA is thus capable of a robust response to burst input from HVC, even at temperatures low enough to substantially suppress tonic spiking.

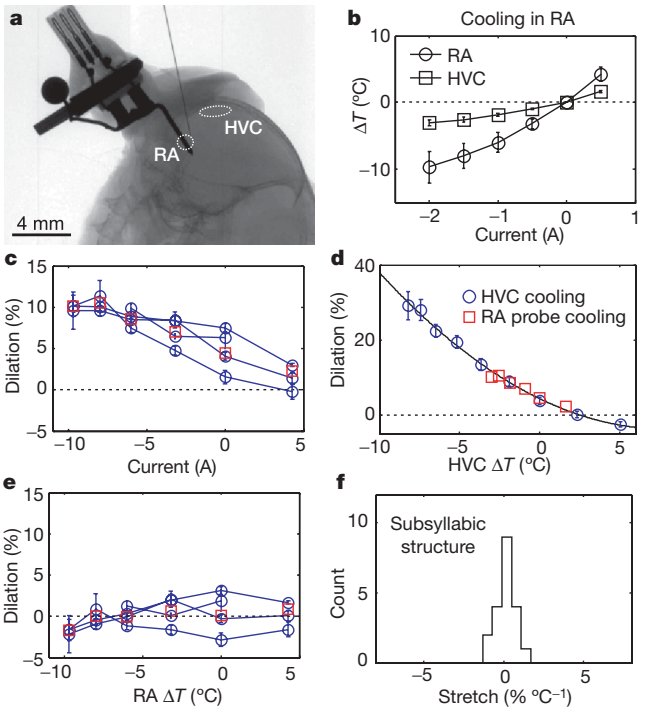

Figure 3 | Effects of RA temperature change on song timing. **a**, X-ray image of the implanted RA cooling device and approximate locations of HVC and RA. **b**, Temperature in RA (200 μm from cooling probe) and HVC as a function of RA probe current. We note that the RA probe produces some cooling in HVC. **c**, Change in motif duration as a function of RA probe current ($n = 4$; red squares, mean). **d**, Average change in motif duration (red squares) during RA probe cooling or heating, plotted as a function of HVC temperature. Also plotted is the average change in motif duration (blue circles) as a function of HVC temperature measured in the HVC cooling experiment (Fig. 1f). **e**, Change in motif duration as a function of RA temperature, corrected for the effect of HVC temperature change. All error bars, s.e.m. **f**, Stretch of subsyllabic elements for the population of RA cooled birds ($n = 4$, 20 syllables), corrected for HVC temperature change.

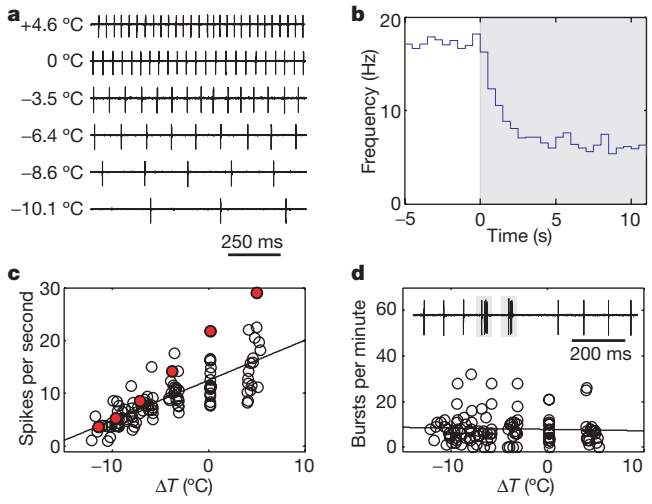

Figure 4 | Effects of RA temperature change on RA spiking activity. **a**, An example of the tonic spiking activity of an RA neuron in an anaesthetized bird for various temperature changes. **b**, Average firing rate response (25 trials) to the application of 1-A cooling current to the RA probe. **c**, Average tonic spiking rate versus temperature for all recorded neurons (19 cells, seven birds). Filled-red circles are from the example shown in **a**. **d**, Spike train showing tonic spiking and spontaneous bursts (top) and incidence of bursts (defined as an instantaneous firing rate greater than 100 Hz) for all neurons (bottom).

The control of song timing by HVC: lateralization

The HVC and RA cooling experiments highlight the centrality of HVC in controlling song timing. HVC is a bilateral structure, and it is natural to wonder how the two HVCs are coordinated during singing. Bilateral multi-unit recordings in HVC have revealed brief episodes of correlated activity across hemispheres that occur before the onset of each syllable (and at some acoustic transitions within long complex syllables)[34,35], probably mediated by feedback pathways from RA to midbrain areas and bilaterally back to HVC[18,19]. Do these episodes reflect actual bilateral synchronization of the HVCs? If the two HVCs were, hypothetically, synchronized only at the beginning of the motif, after which they operated independently, cooling HVC in only one hemisphere (Fig. 5a) should cause the two HVCs to become misaligned in time by more than a whole syllable by the end of the motif (compare control to bilaterally cooled song; Fig. 5c), causing song degradation. However, we found that unilateral cooling of HVC did not produce song degradation, but resulted in slowed songs of normal acoustic structure ($n = 4$; Fig. 5b, c, Supplementary Fig. 10), ruling out any model in which hemispheric synchronization occurs only at motif onsets.

Do both HVCs contribute to song timing? Given previous observations of hemispheric dominance in songbirds[5,36], it is conceivable that one HVC acts as a 'master clock' for song timing and that the other follows as a slave. In fact, in all birds ($n = 4$) we observed that cooling either HVC alone caused song slowing intermediate to that seen for bilateral cooling (Fig. 5b, Supplementary Fig. 10), ruling out the possibility that song timing is controlled by dynamics in a single hemisphere.

One possible explanation for the observation of intermediate slowing is that the left HVC may control the timing of some parts of the song and the right HVC may control the timing of other parts of the song. In fact, we found that cooling the right HVC or left HVC produced less uniform stretching of the song in comparison with bilateral cooling, as predicted by this model (bilateral non-uniformity s.d. of 0.67% $°C^{-1}$, unilateral non-uniformity s.d. of 1.33% $°C^{-1}$; Fig. 5d–j). This inhomogeneity in stretch during unilateral cooling is comparable to the mean stretch (~1.2% $°C^{-1}$), suggesting that a large fraction of song elements were not stretched by unilateral cooling (bilateral cooling, 14% not significantly stretched; left only, 71%; right only, 43%). Furthermore, some elements that were not stretched by right HVC cooling were strongly stretched by left HVC cooling, and vice versa (Fig. 5d–h, Supplementary Fig. 10). Consistent with this, stretch during left cooling was significantly anticorrelated with stretch during right cooling ($P = 0.026$; Fig. 5k; see Supplementary Methods). Thus, it appears that there may be some switching of the control of song timing between the two HVCs on the timescale of song syllables or long subsyllabic elements.

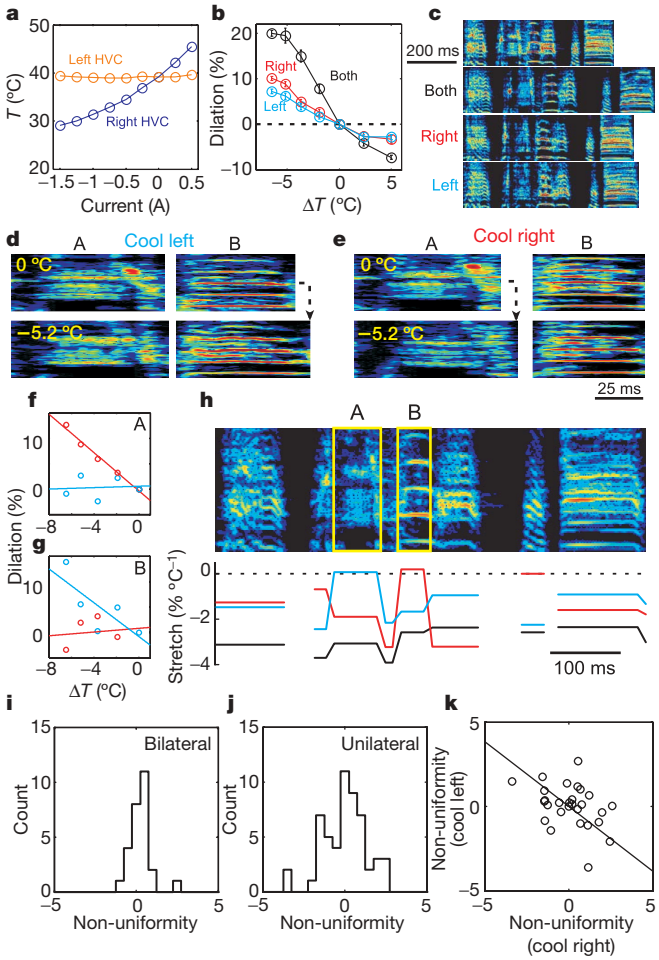

Figure 5 | Effect of unilateral HVC cooling on song timing. **a**, Simultaneous temperature measurements from HVC in both hemispheres when the Peltier device was configured for right HVC cooling. **b**, Change in motif duration as a function of HVC temperature change during unilateral and bilateral cooling in bird no. 11. Error bars, s.e.m. **c**, Spectrograms of song motif during control, bilateral, left and right HVC cooling. **d**, Selective dilation of subsyllabic element B, but not A, during left HVC cooling. **e**, Selective dilation of element A, but not B, during right HVC cooling. **f**, **g**, Dilation of subsyllabic element A (**f**) and B (**g**) during left (blue) and right (red) HVC cooling. **h**, Stretch metric of identified song segments during cooling of left (blue), right (red) or both (black) HVCs. **i**, **j**, Distributions of stretch non-uniformity values during bilateral (**i**) and unilateral (**j**) cooling. **k**, Non-uniformity values during left and right cooling show significant anticorrelation ($P = 0.026$). Solid line shows the first principal component of the distribution.

A chain model of song dynamics

The results of our HVC and RA cooling experiments do not support a view in which the dynamics underlying song timing are divided at different timescales between different brain areas. One possible model of dynamics in the song control system that is consistent with all of our observations is strongly anticipated by the singing-related firing patterns of HVC neurons that project to RA. These neurons burst extremely sparsely during singing, each generating a single brief (~6-ms) burst of spikes at a particular moment in every repetition of the song motif[37]. In addition, different HVC neurons burst at different times throughout the song. We have proposed that, as a population, these HVC neurons form domino-like chains of activity that control the timing of the song[38]. The HVC cooling experiments suggest that the dynamics underlying the sparse sequential activation of HVC neurons reside at least partly within HVC; if the sparse HVC bursts were driven by an independent upstream sequence generating circuit, cooling HVC should not have affected the speed of the song. An interesting possibility is that such sequential chains of activity arise, in part, from a chain-like synaptic organization within HVC[39–43]. In this case, cooling HVC may simply increase the time it takes each neuron to burst following activity in a preceding neuron, thus introducing an accumulating delay that slows down the chain.

Although the cooling results rule out models in which brain areas outside HVC independently or autonomously control song timing on any timescale, they do not exclude the involvement of other brain areas important for song production, in particular feedback projections from RA, through the brainstem/midbrain and Uva back to HVC[19,21]. These projections could form an integral part of the connectivity that generates sequential bursts in HVC. Consider the timescale on which this feedback might operate. In principle, every burst in HVC could be driven by fast, rapidly cycling feedback through this loop, rather than by intrinsic connections within HVC. Cooling anywhere in this feedback loop, including RA, should introduce accumulating delays as well. The fact that we do not observe song slowing from cooling in RA suggests that the feedback circuitry may not

operate at this rapidly cycling timescale, but less frequently during the song.

Thus, one interesting possibility is that HVC may contain multiple independent chains[8] (or modules), which may be associated with syllables or long subsyllabic elements[34]. These modules, each of which could run autonomously by virtue of circuit dynamics within HVC, may then be linked together in time by the feedback connections from RA through the thalamus and back to HVC (Supplementary Fig. 11). The feedback circuitry may act to detect the end of one syllable and rapidly and bilaterally initiate the next gap and syllable, thus simultaneously continuing the song sequence and resynchronizing the two HVCs. In this case, cooling HVC would slow the production of a song syllable, because such production is generated by dynamics within HVC, and would also delay the onset of the next syllable, because this onset is linked to the termination of the previous syllable by feedback circuitry.

A module of chain-like activity in HVC may produce a song syllable as follows. During singing, RA neurons generate a complex but highly stereotyped sequence of spike bursts[14,44]. We have previously suggested that these RA bursts are driven rapidly and on a moment-to-moment basis by bursting inputs from HVC[37,38]. In this view, because RA burst patterns simply follow the timing set by HVC, we would expect RA cooling to have a minimal effect on the timing of RA activity, whereas slowing the chain in HVC would necessarily slow the sequence of bursts in RA. Furthermore, if structures downstream of RA (brainstem motor neurons and syringeal muscles) respond rapidly to descending drive from RA, perhaps on the timescale of the fastest song modulations (10–20 ms)[38],we would expect that slowing the sequence of bursts in RA might slow the song yet have a minimal effect on song acoustic structure. Thus, a simple model of chain-like dynamics in HVC that drives a fast response in RA and downstream structures is consistent with the electrophysiological data and the HVC and RA cooling experiments.

Here we have used local manipulation of brain temperature to identify components within the avian song system that control the timing of a complex behavioural sequence. This approach may be broadly useful in localizing specialized brain circuits that control the timing of other behaviours. We have also used temperature changes to test ideas about the contribution to song production of oscillatory dynamics in the song control pathway, an approach that should be generally applicable to localizing the biophysical origin of oscillatory and other forms of brain dynamics, and for studying their role in brain function.

METHODS SUMMARY

Subjects. Subjects were adult zebra finches (>120 days post-hatch) obtained either from our colony or from an outside distributor (Preferred Birds). All animal procedures were approved by the committee on animal care at the Massachusetts Institute of Technology.

Cooling devices. We used a small (0.7-g) custom-built thermoelectric device based on the Peltier effect to cool HVC and RA. The HVC cooling device was constructed from two 1 mm × 2 mm gold cooling elements that bilaterally contacted the surface of the dura overlying the left and right HVCs. The temperature change in HVC was spatially restricted (Fig. 1c), producing a maximal change of only 0.5 °C in RA, the nearest brain region known to be involved in song production in the zebra finch[5,45]. The current could be switched to flow bilaterally or unilaterally through only the left or only the right cooling element. The RA cooling devices were equipped with a gold spike implanted into RA to facilitate heat transfer.

RA electrophysiology. A craniotomy was made over RA under isoflurane anaesthesia (1.5%). The borders of RA were identified electrophysiologically and the cooling device implanted at the centre. Single neurons were isolated using carbon fibre electrodes (Carbostar-1, Kation Scientific), with a signal-to-noise ratio of greater than 10:1.

Temperature measurements. Temperatures were measured using small thermocouples (5SRTC-TT-K-40-36, Omega). For HVC calibration, three thermocouples were inserted in one hemisphere, under anaesthesia, at respective depths of 0.5, 2.0 and 4.0 mm beneath the gold pad. In some birds, an additional probe was placed in the contralateral HVC (at a depth of 0.5 mm). Once inserted, the thermocouples were secured with dental acrylic and the bird was placed in a cage and allowed to awaken. At each Peltier current level, three minutes were allowed for the brain to reach a steady-state temperature before measurements were taken from all thermocouple locations.

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