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New methods for localizing and manipulating neuronal dynamics in behaving animals

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Where are the 'prime movers' that control behavior? Which circuits in the brain control the order in which individual motor gestures of a learned behavior are generated, and the speed at which they progress? Here we describe two techniques recently applied to localizing and characterizing the circuitry underlying the generation of vocal sequences in the songbird. The first utilizes small, localized, temperature changes in the brain to perturb the speed of neural dynamics. The second utilizes intracellular manipulation of membrane potential in the freely behaving animal to perturb the dynamics within a single neuron. Both of these techniques are broadly applicable in behaving animals to test hypotheses about the biophysical and circuit dynamics that allow neural circuits to march from one state to the next.

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Nearly everything humans learn to do requires the execution of a complex sequence of motor gestures produced in a precise temporal order and with precise timing. Much is now known about how neurons in motor areas spike in relation to individual motor gestures, or even sequences of gestures [1–6], for example directional tuning of neurons in primate motor cortex [7,8] or sequence selectivity of neurons in supplementary motor cortex [9,10]. These are examples of how neuroscientists have begun to decipher how neurons in the brain fire in relation to — or *code for* — complex motor behaviors. But where are the 'prime movers' that drive complex learned behaviors — those circuits that generate the temporal structure of learned behaviors? What controls the order in which states of activity arise and the speed at which they progress? We need to go beyond a description of how neurons fire in relation to behavior to address the circuit and biophysical dynamics that underlie how one state of a neural system is transformed into the next state in time.

In some simple nervous systems, it has been possible to identify small circuits of neurons containing the biophysical dynamics that generate the temporal pattern of a behavior. For example, Stent *et al.* [11••] were able to classify subsets of neurons in the segmental ganglia of the leech as being part of an 'oscillator network' because manipulation of their membrane potential altered the timing of the rhythmic swimming behavior. In contrast, manipulating other – 'follower' – neurons had no effect on timing because their activity is simply driven by the oscillator network. Here we say that the dynamics that underlie the swimming behavior are located entirely within the oscillator network, even though the follower neurons also exhibit time-dependent activity necessary for swimming.

Addressing these questions for more complex behaviors in large brains with many more neurons is more difficult. Lesions or pharmacological inactivation can tell us whether a brain region is necessary for the expression of a particular behavior. Electrophysiological recordings can tell us whether activity in a brain area is correlated with a behavior. Electrical stimulation can tell us whether activation of a region (or the axons passing into or through that region) can disrupt or elicit a behavior [1,12], but none of these approaches can reveal whether the biophysical dynamics within a region are actively involved in timing a behavior.

Here we describe two techniques that have recently been applied to the question of the origin of biophysical dynamics in neural circuits: localized brain cooling and the intracellular manipulation of neuron membrane potential. Using these techniques, hypotheses can be tested about the dynamical origin of temporal structure in neural circuits that could not be tested using other techniques. We will focus on the application of these techniques to examine the origin of song timing in a songbird, an excellent model organism in which to address questions about the neuronal dynamics underlying complex learned sequences in the vertebrate brain. The song is mediated by a discrete set of motor nuclei, known as the song motor pathway, that are necessary for the production of adult song (Figure 1a) [13–17]. One

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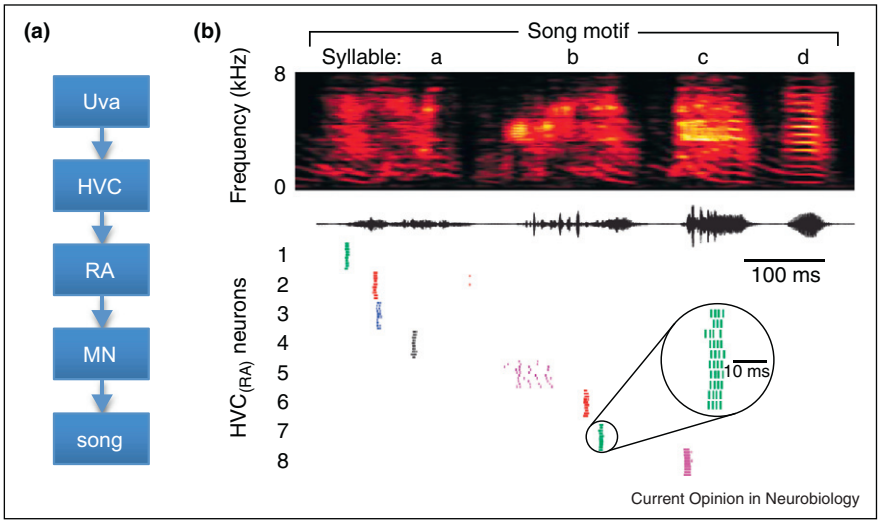
Figure 1



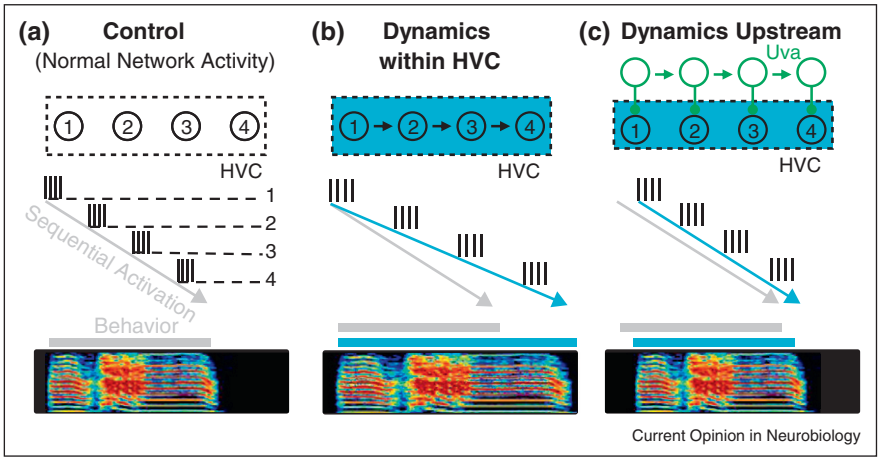
Sequential activity in the songbird motor pathway. **(a)** Block diagram of brain regions involved in the production of song. Abbreviations: Uva, nucleus uvaeformis; HVC, used as a proper name; RA, robust nucleus of the archistriatum; MN, brainstem motor nucleus. **(b)** Raster plot of the spiking patterns of eight RA-projecting HVC neurons recorded during singing. Each row of tick marks shows spikes generated during one rendition of the song; roughly ten renditions are shown for each neuron. The neurons were identified by antidromic stimulation from RA. The song spectrogram is shown at top. Note that each RA-projecting HVC neuron bursts reliably at a single precise time in the song.

nucleus, known as HVC (used as a proper name), is a cortical premotor brain region containing neurons that generate a single burst of spikes at a particular moment in the song. As a population, these neurons form a sparse sequential representation of time, or temporal order, in the song [18] (Figure 1b).

Using temperature to localize neuronal dynamics

Where are the dynamics that underlie the generation of sparse sequential bursts in HVC (Figure 2a)? One possibility is that the dynamics are contained entirely within HVC (Figure 2b). For example, one could envision a

Figure 2



Localizing dynamics underlying sequence generation in HVC. **(a)** Sequential bursts within HVC drive precise sequences of activity in the downstream motor pathway that produce song behavior. **(b)** In Model 1 sequences are generated by circuitry within HVC. Slowing circuit dynamics in HVC should therefore slow down the sequence of bursts, thus slowing the song. Grey and blue arrows indicate speed of control and cooled burst sequence, respectively. Bars below indicate duration of control and manipulated song syllables. **(c)** In Model 2 the dynamics underlying song timing reside upstream of HVC, for example in Uva. HVC bursts are triggered by timing signals from Uva. Slowing circuit dynamics in HVC might increase the latency of the HVC response to Uva input but this latency increase will be the same for every burst. (Increased latency represented by rightward shift of blue arrow.) Thus, the number of bursts per unit time will not change and the song will not slow down.

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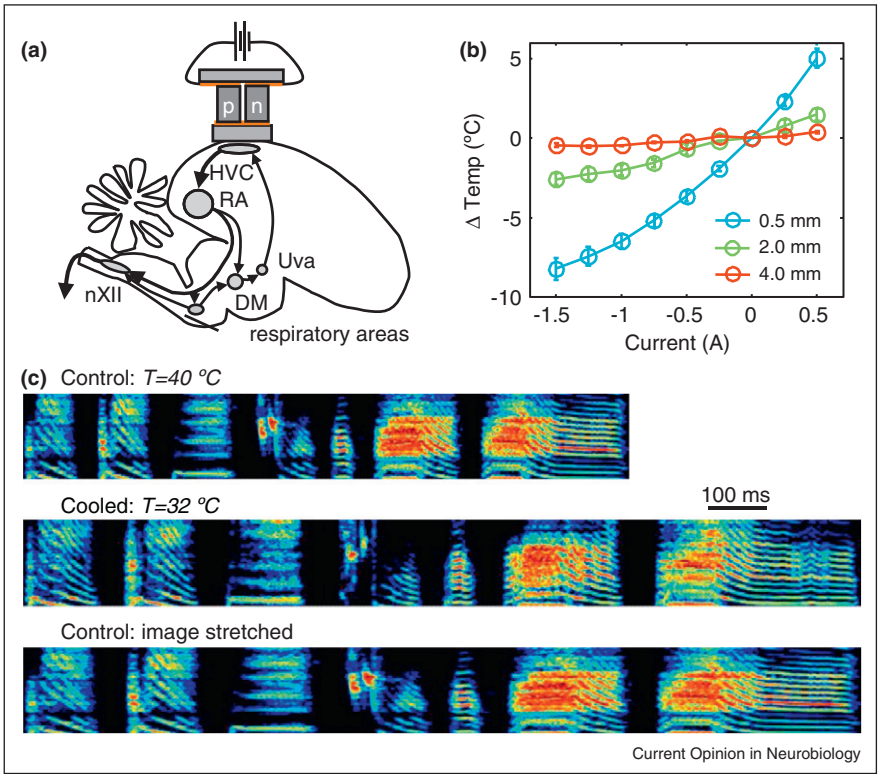
chain of activity in which each neuron is activated by a previous group of neurons and in turn activates the next group. An alternative possibility is that the bursting in HVC is directly driven by synaptic inputs from upstream brain areas, such as the thalamic nucleus Uva [16,17,19]. In the latter case, the timing of HVC activity could be controlled entirely by circuitry upstream of HVC (Figure 2c).

These two models give very different predictions for what would happen if we could slow down the biophysical processes in HVC. Imagine we could inject some sort of 'neuronal molasses' into HVC that does not inactivate the circuit, but just makes all neuronal processes move a little more slowly. If the circuitry and biophysical processes that control song timing reside entirely within HVC, then the time it takes each neuron to activate the next neuron in this sequence should increase. There should therefore be fewer bursts per unit time, and the song should slow down (Figure 2b).

The prediction is quite different if the burst sequence in HVC is driven by timing inputs from upstream of HVC. Slowing HVC could increase the duration of HVC bursts and would increase the time it takes HVC neurons to respond to this upstream input, but this increased latency would be the same for every HVC neuron. Thus, there would be the same number of bursts per unit time, and the song would not slow down (Figure 2c).

Temperature change is a useful manipulation to distinguish hypotheses like these about the role of specific circuits in timing of a behavior. Because it affects many neuronal processes such as synaptic transmission, axonal conduction velocity, and spiking properties [20–24], temperature change can be used very generally, even without detailed knowledge of the underlying circuit or neuronal properties. Although large decreases in temperature ($\Delta T > 30^{\circ}C$) can result in inactivation [25,26], slight cooling ($\Delta T < 8^{\circ}C$) can slow circuit activity. Indeed, there are several examples now in which small temperature changes in simple central pattern generators have been shown to produce substantial changes in oscillatory cycle time [27,28••,29] but otherwise leave the circuit and behavioral function intact [28••,30•,31,32].

Figure 3



Slowing dynamics with local temperature change. **(a)** Schematic of the zebra finch brain highlighting the motor pathway and showing the placement of the thermoelectric cooling device over HVC. **(b)** Bidirectional temperature changes at a variety of depths under the surface of the thermoelectric probe, as a function electrical current through the device. Note that the temperature changes are localized to within 1–2 mm of the cooling device: The distance to the center of HVC is ~0.5 mm and to the center of RA is ~4 mm. **(c)** Cooling HVC produces a slowing of the song across all timescales. Shown are song spectrograms from a bird with HVC at normal brain temperatures (top) and during cooling of HVC by 8°C (middle). At bottom is a linearly warped image of the normal song spectrogram.

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Temperature changes can be applied locally to distinguish the influence of even nearby brain areas, such as nucleus RA and HVC in the song motor pathway. The combination of thermal diffusion and local perfusion of the brain with a warm blood results in a localized effect of brain cooling. In birds, and probably mammals, the temperature change falls off from a cooling surface with a length constant of about 1.0–1.5 mm [33]. Local cooling can be applied at the surface of the brain (for example, neocortex), or to structures deep within the brain using insulated probes. Using this approach, we developed a miniature device using small solid-state electronic heat pumps to provide local cooling of specific brain regions in the free-behaving, singing zebra finches [33,34] (Figure 3a,b). We found that bilateral cooling of HVC led to a nearly uniform slowing, or stretching, of the song by about 3% per degree of temperature change (Figure 3c). This stretch of the song occurred at all timescales: detailed acoustic structure within song syllables, the interval between syllable onsets, and the interval between song motifs. This finding provides strong evidence against models in which the timing of song vocalizations, on any timescale, is controlled by circuitry outside of HVC, and suggests that HVC may function as the 'high vocal clock' of song motor sequences.

Local temperature changes can be especially powerful when used to distinguish the roles of multiple interacting brain areas. If cooling one of these areas produces a slowing of the behavior while another does not, then strong conclusions can be drawn about the lack of involvement of the area in which cooling has no effect. For example, while cooling HVC causes a slowing of song structure, temperature changes in RA, the structure in the motor pathway to which HVC projects, did not result in a measurable change in song timing [33]. Taken together, these results suggest that biophysical dynamics within RA play little role in the control of song timing.

Observing an effect on timing from cooling a brain area may provide some evidence that timing originates in that area, however one must be careful to rule out other possible interpretations. For example, consider a situation in which the dynamics in one circuit (area B) are strongly sensitive to the amount of tonic 'activation' or perhaps neuromodulatory input from an upstream area (area A). Because cooling can substantially affect the firing rate of tonically active neurons (see Ref [33] for an example), then cooling upstream area A could affect the temporal patterns in the downstream circuits, even though biophysical dynamics in area A are not directly involved in generating temporal patterns of activity. As with any technique, combining cooling with other approaches, such as electrophysiology, will be important to dissect neural circuit dynamics.

Manipulating intracellular membrane potential during behavior to test circuit models

Once HVC was localized as a principal locus of timing in the song pathway, it was then possible to address the detailed circuit mechanisms that underlie the sparse sequential activation of neurons in this area. Two major classes of models have been proposed to explain sequence generation within neural circuits. Groups of neurons may be linked together with specific local connections such that activity can propagate, wave-like, through a synaptically connected 'chain' of neurons (Figure 4a, chain model) [35–39]. Alternatively, sequences could be generated in the absence of overt feed-forward connections between neurons. For example, a population of neurons could exhibit a gradual and global ramping-up of membrane potential such that the most excitable neurons reach threshold first, while the least excitable neurons reach threshold later (Figure 4b, ramp-to-threshold model). Such global modulation of membrane potential is thought to underlie hippocampal theta sequences during theta activity [40] and perhaps replay sequences during sharp-wave activity [41,42]. In these models, the diversity of neuronal excitability (which controls the time at which each neuron spikes in the sequence) could be a function of intrinsic cellular excitability [43,44,45•], or of recurrent network excitation [41].

Slow subthreshold inhibitory dynamics on the timescale of song syllables (~100 ms) has been observed in HVC *in vitro* [46], raising the possibility that such a ramp-to-threshold circuit could control burst timing in HVC. Note that any 'ramping' mechanism produced by biophysical dynamics within HVC would also likely be slowed by HVC cooling, so that the ramp-to-threshold model would predict a slowing of the song sequence by HVC cooling. Thus, the ramp-to-threshold model and the chain model described above would be very difficult to distinguish based on evidence from spike data or cooling experiments.

However, these two models yield very different predictions for the pattern of subthreshold activity that one would observe during singing. Specifically, in the ramp-to-threshold model, the membrane potential of each HVC neuron should exhibit a slow depolarizing ramp in the hundreds of milliseconds before burst onset, whereas in the chain model, each burst should be preceded by a fast depolarizing input from previously active neurons. Also, intracellular injection of hyperpolarizing current would be expected to make the recorded neuron reach threshold later, delaying the onset of the burst. Likewise, injection of depolarizing current would be expected to advance the time of the burst.

In order to test these predictions of these two circuit models, we adopted an approach recently introduced for intracellular recordings in the freely moving rat [47•,48] to carry out intracellular recordings in unrestrained, singing

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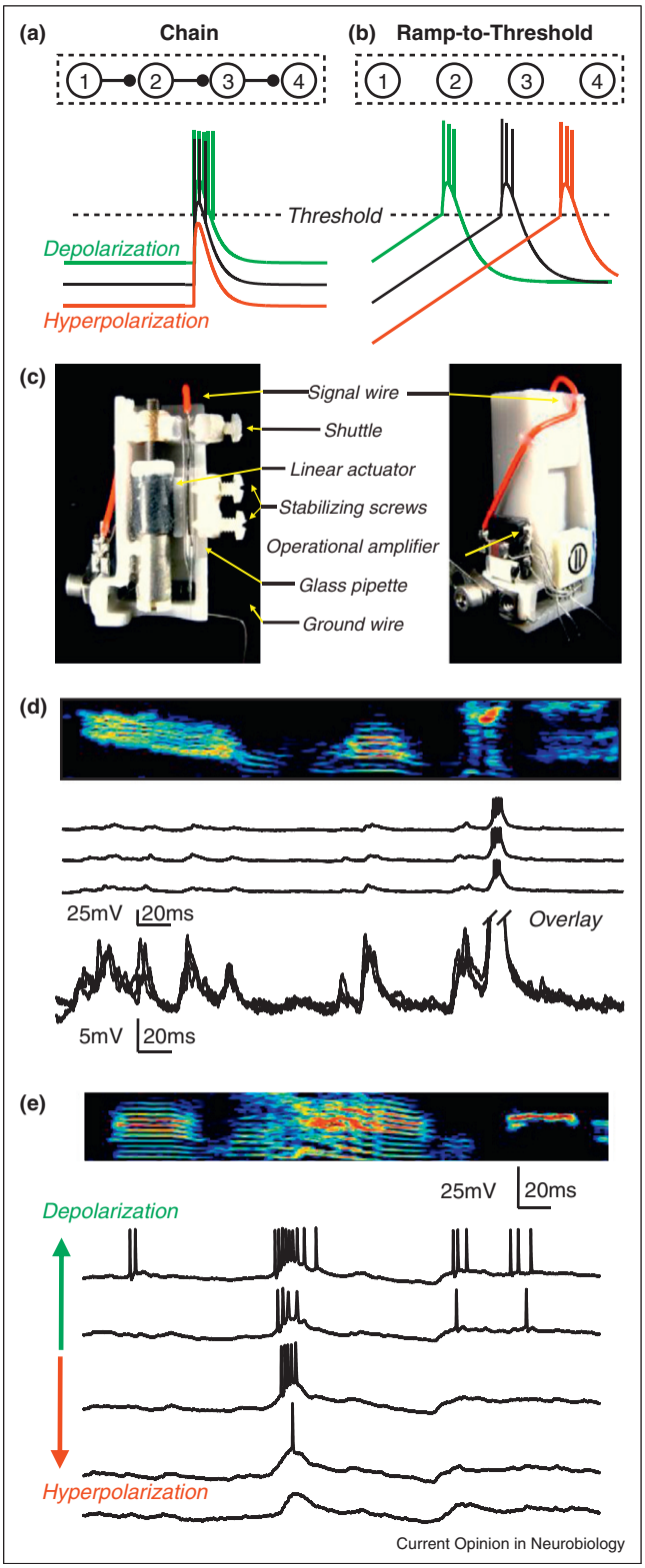
Figure 4



Testing circuit dynamics during natural behaviors with intracellular current injection and recording. **(a)** In the chain model of sequence generation, neurons activate each other sequentially through sparse feed-forward synaptic connections. This model predicts that intracellular current injection should have little effect on burst timing. Strong hyperpolarizing currents (red) might suppress spiking, but there should be a subthreshold depolarizing potential at the time of the burst. **(b)** In the ramp-to-threshold model, neurons are activated by a global ramp of excitation. The time at which each neuron spikes is set by its intrinsic excitability. This model predicts that burst timing should be delayed by hyperpolarizing current (red) and advanced by depolarizing current injection (green). **(c)** Motorized microdrive for intracellular recording and current injection in unrestrained, freely behaving animals. **(d)** Intracellular recording of a single RA-projecting HVC neuron during singing. The membrane potential is aligned to the song motif. Traces are shown at low and high voltage gains (top and bottom, respectively). Note the absence of slow membrane potential ramping before burst onset. **(e)** Consistent with the chain model, burst timing was unaffected by steady current injection. Note the depolarizing potential at the time of the burst in the most hyperpolarized condition (bottom).

zebra finches. We developed a small (1.6 g) microdrive and miniature headstage preamplifier for use with sharp glass microelectrodes (Figure 4c). With this device we were able to record synaptic inputs as well as spiking outputs from antidromically identified premotor neurons in HVC (Figure 4d). The results were clearly consistent with the predictions of the chain model — in no cases did HVC neurons exhibit a ramping-up of membrane potential in the hundreds of milliseconds before burst inset. Indeed, bursts were preceded by a rapidly rising depolarizing potential within 5–10 ms before burst onset, consistent with the idea that neurons are being driven by input from a previous 'link' in a chain of neurons. Furthermore, hyperpolarizing or depolarizing current injection did not change the time of the burst onset by more than a few milliseconds (Figure 4e), inconsistent with the idea that the time at which a neuron bursts is controlled by a slow ramping up to spike threshold.

Summary and conclusions

Here we describe two approaches recently applied to localizing and characterizing the neuronal dynamics underlying the generation of complex behavioral sequences in behaving animals. While we have framed these ideas in terms of the circuits underlying behavior, the question of neuronal dynamics is pervasive in all aspects of brain function that involve time-dependent computations [49]. The tools we describe here should be broadly applicable to the study of brain dynamics underlying temporal selectivity [50•], changes in attention [51•], motor planning [52], and the generation of theta rhythm and phase precession important for hippocampal function [41,53••,54]. Other potential applications include examining the rich dynamics underlying the control of intracortical information flow [55•] and spontaneous brain states [56•], or even localizing the dynamics underlying pathological states of brain activity such as Parkinsonian tremor [57•].

The tools we review here are complementary to recent approaches allowing optical control of activity in specific sets of neurons [58–66] or the targeting of ligand-gated channels to genetically specified sets of neurons in a

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circuit [67–70,71••]. These approaches will primarily be useful for studying neural circuits in which neuronal populations can be targeted with genetic tools, and about which enough is understood to formulate hypotheses about the function of specific circuit elements. In contrast, the broad effectiveness of localized brain cooling across neuronal subtypes and biophysical processes within a circuit make this approach perfectly suited for the initial stage of cracking circuit dynamics—localizing within a set of interconnected brain regions where the neuronal dynamics are that underlie a behavior or a computation. Intracellular recording and current injection are similarly broadly applicable to address detailed questions about the dynamics of neuronal circuits in behaving animals.

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