Spontaneous Dynamics of Rhythmic Spinal Interneurons Measured With Two-Photon Calcium Imaging and Coherence Analysis

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Kwan AC, Dietz SB, Zhong G, Harris-Warrick RM, Webb WW. Spontaneous dynamics of rhythmic spinal interneurons measured with two-photon calcium imaging and coherence analysis. J Neurophysiol 104: 3323–3333, 2010. First published September 22, 2010; doi:10.1152/jn.00679.2010. In rhythmic neural circuits, a neuron often fires action potentials with a constant phase to the rhythm, a timing relationship that can be functionally significant. To characterize these phase preferences in a large-scale, cell type–specific manner, we adapted multitaper coherence analysis for two-photon calcium imaging. Analysis of simulated data showed that coherence is a simple and robust measure of rhythmicity for calcium imaging data. When applied to the neonatal mouse hindlimb spinal locomotor network, the phase relationships between peak activity of >1,000 ventral spinal interneurons and motor output were characterized. Most interneurons showed rhythmic activity that was coherent and in phase with the ipsilateral motor output during fictive locomotion. The phase distributions of two genetically identified classes of interneurons were distinct from the ensemble population and from each other. There was no obvious spatial clustering of interneurons with similar phase preferences. Together, these results suggest that cell type, not neighboring neuron activity, is a better indicator of an interneuron’s response during fictive locomotion. The ability to measure the phase preferences of many neurons with cell type and spatial information should be widely applicable for studying other rhythmic neural circuits.

INTRODUCTION

In rhythmic neural circuits, the spike-oscillation phase is an important quantity that relates directly to function. For example, in the spinal cord, there are several identified interneuron types that have been shown to fire at specific phases of the locomotor cycle. Some interneuron types fire preferentially in the ipsilateral motor output phase, such as those identified by expression of the EphA4 receptor (Butt et al. 2005) or the transcription factor Hb9 (Hinckley et al. 2005), by cholinergic innervations to motoneurons (Zagoraiou et al. 2009), and GABAergic interneurons in the dorsal horn (Wilson et al. 2010). Other cell types prefer the contralateral phase, such as Renshaw cells (Nishimaru et al. 2006), or can fire in either ipsilateral or contralateral phases, such as subclasses of commissural interneurons (Butt and Kiehn 2003) and V2a interneurons expressing the transcription factor Chx10 (Dougherty and Kiehn 2010; Zhong et al. 2010). These firing preferences, along with complementary data, have been used to speculate on the functional roles of the different neuronal classes in the central pattern generator network for locomotion. However, most studies used single cell electrophysiology, where cells are difficult to target in the intact preparation. With this method, one cannot record multiple neurons (identified or otherwise) simultaneously to compare their patterns of activity.

Large-scale two-photon calcium imaging overcomes these limitations by simultaneously recording the somatic calcium transients from many neurons (Stosiek et al. 2003) whose cell type can be identified by genetically encoded fluorescent markers (Wilson et al. 2007). Two-photon microscopy is more suitable for deep imaging under the surface of intact tissue preparations (Denk et al. 1990; Helmcen and Denk 2005), which is often essential for rhythmic neural circuits to remain functional. For these reasons, there have been numerous studies using two-photon calcium imaging to examine rhythmic pattern generator circuits such as those driving locomotion in the spinal cord (Kwan et al. 2009; Wilson et al. 2007) and the medullary respiratory network (Hayes and Del Negro 2007; Winter et al. 2009). However, because somatic calcium is a slow, filtered measurement of spiking activity, the exact spike train often cannot be reconstructed with high fidelity and temporal precision. Common techniques for spike inference are ill suited for analyzing rhythmic neurons, which have high firing rates. Therefore the development of a simple, quantitative method to analyze rhythmic calcium imaging data would aid in the interpretation of the experimental results.

Here, we show that coherence analysis is highly effective when applied to large-scale calcium imaging for quantifying the rhythmicity and preferred phase of a large number of cells. Coherence is a frequency-domain quantity that reflects how well two signals track each other at specific frequencies. In an early application to motor physiology, coherence was used to characterize the synchronization of magnetoencephalogram (MEG) and EMG (Conway et al. 1995; Rosenberg et al. 1989). More recently, coherence has been used to quantify the coupling of ventral root outputs during fictive locomotion (Miller and Sigvardt 1998; Mor and Lev-Tov 2007). Coherence has also been widely applied to quantify the coupling of single- or multi-unit activity and the local field (Womelsdorf et al. 2006) and between local fields from different brain regions (Bragin et al. 1995). For imaging, coherence has been applied to voltage-sensitive dye studies to investigate the functional connectivity of cell pairs, by driving one cell with a sinusoidal current (Cacciato et al. 1999; Taylor et al. 2003). In this study, we showed that coherence can be used to analyze large-scale rhythmic calcium imaging data and applied it to study the activity of interneurons in the neonatal mouse hindlimb spinal locomotor circuit. We characterized the phase preferences of ventral spinal interneurons relative to the motor output during fictive locomotion, focusing on the effects of the neuron’s cell type identity and spatial location.

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METHODS

Calcium imaging and electrophysiology

All experimental procedures were performed in accordance with the Cornell University Institutional Animal Care and Use Committee guidelines. We prepared and imaged in vitro neonatal (P1–P3) spinal cords (n = 10) of mice from three transgenic lines: Hb9-GFP (Hinckley et al. 2005; Wilson et al. 2005), Hb9-GFP/lacZ (Kwan et al. 2009), and Chx10-CFP (Zhong et al. 2010). The procedures for the dissection and two-photon calcium imaging have been described elsewhere (Kwan et al. 2009). Briefly, Fluo-3 AM calcium indicator dye was pressure-injected into the upper lumbar (T13–L2) ventromedial spinal cord. Fictive locomotion was induced by bath application of serotonin (5-HT), N-methyl-d-aspartate (NMDA), and dopamine (DA) in micromolar concentrations. Ipsilateral and contralateral flexor motor outputs were recorded extracellularly from T13, L1, or L2 ventral roots using glass suction electrodes. It has been shown that the predominant motor output from these ventral roots is flexor-related. Thus activity of our T13–L2 interneurons in phase with the ipsilateral ventral roots will be flexor-related, whereas activity in phase with the contralateral ventral roots will be related to ipsilateral extensor motoneuron activity. We did not image interneuron activity more caudally (L5–L6), where the predominant ipsilateral motoneuron output is extensor-related. After two-photon calcium imaging, the Hb9-GFP/lacZ spinal cords were whole mount stained with X-gal to identify cells that expressed LacZ (Kwan et al. 2009). Interneurons with both GFP and LacZ expression were deemed to be true Hb9 interneurons and used for the cell type–specific analysis. Each time-lapse fluorescence and ventral root recording lasted ~50 s. Almost all of the time-lapse images consisted of 480 frames, acquired at 9.2 Hz with bi-directional scanning. Three trials were acquired consecutively at each field of view, which on average contained 23 ± 1 cells located in the same z-plane.

Neurons in the ventromedial region (lamina VIII-X) were imaged in the intact cord from the ventral surface. To image the more dorsally located intermediate lamina VII, which contains the Chx10 interneurons and other cells, the dorsal surface of the spinal cord was locally removed from the L2 lumbar segment (Zhong et al. 2010). Dye injection and imaging were performed from the cut dorsal surface. For ventromedial region imaging in the intact cord, fictive locomotion was elicited by 9 μM NMDA, 12 μM 5-HT, and 18 μM DA, whereas for intermediate lamina VII imaging in the dorsal horn–removed preparation, 6 μM NMDA and 9 μM 5-HT were used. In the latter case, only NMDA/5-HT were used because cycle periods needed to be more variable for other experiments (Crone et al. 2009), and the addition of DA tended to stabilize the cycle period. In the data presented here, the mean locomotor frequency was slower for the first peak; this forced a long interpeak time relative to the expected decay time constant to avoid overlapping peaks. The isolated calcium peaks were normalized in amplitude and aligned in time by maximum F(t). From 30 cells and a total of 69 isolated calcium peaks, we fitted the mean F(t) of each cell with an exponential decay function. The time constant for calcium signal decay was 1.6 ± 0.1 (SE) s (Fig. 1B). Our measured value is slower than the ~0.5–1 s measured in other studies that used bolus-loaded calcium indicators, such as in neocortex in vivo (Garaschuk et al. 2006). This difference could arise because our in vitro experiments were performed at room temperature and could also arise from different calcium buffering properties in spinal versus cortical neurons.

Voltage clamp recordings

For each cell in each trial, the change in fluorescence, ΔF(t), was calculated as the percent change from baseline, so \( \frac{\Delta F}{F} = \frac{F(t) - f_0}{f_0} \), where F(t) is the raw fluorescence pixel sum and f_0 is the minimum of f(t). The value of f_0 might overestimate the true baseline calcium level, which is not measurable in rhythmically active neurons that are never at rest. Extracellular recordings of motoneuron activity in the ipsilateral and contralateral ventral lumbar roots were rectified in the post hoc analysis to obtain the absolute motor output signal.

In some experiments, no neurotransmitters were added, and the spinal cord was superfused only with Ringer solution. Fictive locomotion was absent, and the neurons had sparse spontaneous activity (Fig. 1A), which allowed us to accurately measure the decay rate of the calcium signal associated with action potentials. To conservatively isolate single events, calcium peaks were detected by thresholding the calcium signal associated with action potentials. To conservatively isolate single events, calcium peaks were detected by thresholding the calcium signal associated with action potentials. To conservatively isolate single events, calcium peaks were detected by thresholding the calcium signal associated with action potentials. To conservatively isolate single events, calcium peaks were detected by thresholding the calcium signal associated with action potentials.

Multitaper coherence analysis

Discrete Fourier transform evaluates the frequency components contained within a discretely timed function (the recording) that samples a continuous signal (the underlying neural signal). This transform is accurate if the sampling rate exceeds twice the signal frequency and if the function is exactly periodic. The periodicity condition arises because discrete Fourier transform can be thought of as repeating the finite-length recording and pasting it end-to-end periodically to construct one infinite-length function to be fitted by sinusoids of different frequencies. Because real recordings are not exactly periodic, before the discrete Fourier transform, the recording is often smoothly truncated at the ends by filtering with the so-called...
window function, also known as a taper. There are many types of tapers that minimize different kinds of errors (Oppenheim and Schafer 1989). One framework, multitaper spectral analysis (Thomson 1982), defines and uses a set of orthonormal tapers for calculating independent estimates of the Fourier transform to generate an optimal, weighted result and a bootstrap error. The multitaper framework has been developed extensively for analyzing neural data (Kleinfeld and Mitra 2010; Mitra and Bokil 2008; Mitra and Pesaran 1999).

In the application for calcium imaging of a rhythmic neural circuit, we are interested in the interaction between the single cell fluorescence signals, $F(t)$, with the output response of the entire circuit, $R(t)$. In the specific case of the spinal cord, the output response is the rhythmic bursts recorded from motoneuron axons in the lumbar ventral roots. The multitaper power spectrum $S(f)$ of the circuit output $R(t)$ is given by

$$S(f) = \frac{1}{K} \sum_{k=1}^{K} \left| R_k(f) \right|^2$$

where $K$ is the number of tapers used and $R_k(f)$ is the Fourier transform calculated using the $k$th taper with the discrete Fourier transform

$$R_k(f) = \frac{1}{N} \sum_{n=1}^{N} R(t) e^{-i2\pi nf_k(t)}$$

where $N$ is the number of samples and $w_k(t)$ is the $k$th-order taper function as defined by Thomson (1982). We select the largest, non-zero peak in the power spectrum as the rhythmic circuit’s fundamental frequency, $f_o$. The coherence $C(f)$, defined as the normalized cross-spectrum of two functions, measures how well the two functions track each other at a specific frequency. For the fluorescence $F(t)$ and the circuit response $R(t)$, the coherence $C(f)$ is

$$C(f) = \sqrt{\frac{\frac{1}{K} \sum_{k=1}^{K} |F_k(f)|^2 \cdot \frac{1}{K} \sum_{k=1}^{K} |R_k(f)|^2}{\frac{1}{K} \sum_{k=1}^{K} |R_k(f)|^2}}$$

Coherence is a complex function of frequency; at the circuit fundamental frequency, the magnitude $|C(f = f_o)|$ and the phase $\theta(f = f_o)$ of peak neuronal activity relative to the network output are extracted.

The analysis was implemented in 64-bit MATLAB with the multitaper spectral analysis package Chronux (www.chronux.org) (Mitra and Bokil 2008). Using more tapers leads to more weighted estimates for a more robust calculation of $C(f)$ and $\theta$. However, more tapers reduce the effective duration of $F(t)$, so information about low frequency components is lost. This relationship between the number of tapers, $K$, and the frequency resolution, more specifically the time-bandwidth product, can be quantified (Kleinfeld and Mitra 2010)

$$K = 2(T\Delta f) - 1$$

where $T$ is the duration of the recording and $\Delta f$ is the frequency resolution. Our imaging experiments typically ran for $\sim50$ s, and the locomotor frequency was greater than $\sim0.2$ Hz, so we chose to use nine tapers for a frequency resolution of $0.1$ Hz, ensuring that the DC and the circuit frequency components did not mix.

Because the slow temporal dynamics of somatic calcium handling and dye buffering was approximately a linear filter onto the underlying spiking activity, it would introduce an offset in coherence phase. If uncorrected, the fluorescence signals would additionally lag the motor output. We corrected for this bias by calculating the expected offset using simulated fluorescence and circuit output. The simulated $R(t)$ is a 0.23 or 0.35 Hz sine function with an amplitude offset, and the simulated $F(t)$ is a 0.23 or 0.35 Hz spike train convolved with an exponentially decaying function with a time constant of 1.6 s. The output frequency value of 0.23 Hz or 0.35 Hz and time constant of 1.6 s were chosen to match the values of motor frequency and calcium decay time constant experimentally measured in mouse spinal cord in vitro. We will discuss the calculation and validity of the offset subtraction, along with tests to verify that the offset is constant under various conditions, in RESULTS.

**Spike inference from calcium**

We examined two common methods to infer spikes from calcium: thresholding and template matching. For the simplest form of thresholding, a spike is inferred when the local maximum of fluorescence $F(t)$ rises above a certain value, the threshold. Because a single threshold is sensitive to noise and drifts in calcium signal, spikes were inferred when the change of $F(t)$, from the preceding local minimum to the current local maximum, is greater than a threshold. The choice of threshold affects the balance between detection rate and false positives, which we manually balanced; we chose a threshold of 3 SD above the mean $F(t)$. For template matching, a user-defined template is designed to match and identify the sharp-rising, slow-decaying calcium transients caused by a spike. Matching is done by filtering $F(t)$ with the user-defined template and inferring a spike when the local maximum of the filtered function is above a threshold. We used a template with duration of 2 s that consisted of 0.5 s of zeros followed by 1.5 s of an exponential decay with a shape that was chosen to exactly match the simulation. The duration of the template was chosen considering that a longer template leads to a more robust inference; however, duration needs to be less than the expected interspike interval to avoid matching multiple peaks at once, i.e., less than the period of $R(t)$. Again, the choice of threshold for evaluating the filtered function affects the balance between detection rate and false positives, which we manually balanced for this case to be 1 SD above the mean filtered $F(t)$. The timing between the inferred spikes and simulated response $R(t)$ were quantified using circular statistics, commonly used for characterizing spike-motor output phase relationships (Berkowitz and Stein 1994; Kjaerulff and Kiehn 1996). The fundamental circuit frequency, $f_o$, was specified in the simulation so the phase of the oscillatory $R(t)$ was known precisely at each time point. The spike-triggered phases of $R(t)$ were averaged vectorially to yield a mean vector whose angle was the preferred phase of firing and whose magnitude, called the $r$ value, reflects rhythmicity.

**Statistics**

We calculated the $|C|$ confidence level, $|C|_{P=0.05}$ (Kleinfeld and Mitra 2010), where the probability of observing a coherence magnitude that exceeds $|C|_{P=0.05}$ is 0.05, given the null hypothesis. In other words, a coherence magnitude above $|C|_{P=0.05}$ is statistically significant with a $P$ value of 0.05. The actual value of $|C|_{P=0.05}$ depends on the number of tapers and the number of trials. Uncertainties for $\theta$ and $|C|$ were calculated using Jackknife resampling and plotted to give 95% CIs for the estimates.

All phase angles were presented in units of radians. For circular data, such as the coherence phase, the mean values and statistical tests were calculated using the MATLAB CircStat toolbox (Berens 2009). Mean values are reported with SE. The significance of a mean phase was assessed by the Rayleigh test. Two means of coherence phases were compared using the Watson-Williams test (Zar 1999).

**RESULTS**

Two-photon calcium imaging of spinal interneurons during fictive locomotion

We used two-photon calcium imaging to record the somatic calcium transients of cells in the upper lumbar segments ($T_{12}$–$L_{2}$) of the mouse spinal cord in vitro (Fig. 2, A–D).
Pressure injection of Fluo-3 AM into the ventromedial region of the spinal cord labeled cells indiscriminately, but because we always imaged medially near the midline, the imaged cells were primarily consisted of interneurons and some glial cells, but not motoneurons. The absence of motoneurons in our data were confirmed in Hb9-GFP mice because the more lateral motoneuron column could be identified as a row of large GFP-expressing somata. Calcium transients in spinal interneurons are directly related to spiking activity, as was previously shown by concurrent imaging and whole cell recording (Kwan et al. 2009). Fictive locomotion, elicited by NMDA/5-HT/DA application, was characterized by rhythmic, alternating bursts of motoneuron activity in vitro. Motor activity was recorded extracellularly from ipsilateral and contralateral upper lumbar (T13–L2) ventral roots, which have been shown to be primarily flexor related (Whelan et al. 2000). The imaged cells were located within one lumbar segment of the recorded lumbar ventral root. The fluorescence signal of a typical interneuron resembled a noisy sinusoidal function with no clear rise and decay (Fig. 2E), suggesting that most spinal interneurons had high firing rates relative to the calcium decay constant (i.e., approximately >1/1.6 s = 0.6 Hz). To analyze this complex rhythmic circuit and imaging data, we calculated the coherence between the fluorescence and the motor output.

Coherence analysis of calcium imaging data from a rhythmic neuron

An intuition for understanding how coherence applies to calcium imaging data can be developed by considering a simplified scenario: simulated fluorescence recorded from a model neuron that fires once at the peaks of the circuit’s oscillating output (Fig. 3A). Power spectra and coherence were calculated using multitaper spectral analysis (see METHODS). As expected, the power spectra of the fluorescence and output have peaks at the circuit frequency along with higher frequency harmonics (Fig. 3B). The coherence, defined as the normalized cross-spectra of the fluorescence and the circuit output, quantifies how well the signals couple at each frequency. The coherence magnitude (Fig. 3C), bounded by 0 (neuron is not rhythmic) and 1 (neuron is completely rhythmic with the circuit output), is at or near unity at the fundamental and
harmonic circuit frequencies, indicating that the fluorescence is rhythmic and has a fixed phase relationship with the oscillating circuit output. The coherence phase (Fig. 3C) quantifies this phase preference, plus an offset caused by the slow somatic calcium dynamics. In this example, there is no time lag between the peak calcium activity of the neuron and the circuit output so the nonzero coherence phase of 1.14 in the right panel of Fig. 3C is entirely caused by the slow offset of the calcium signal. Because we are extracting coherence magnitude and phase at a single frequency (i.e., the circuit fundamental frequency), the analysis is linear, and this offset can be simply subtracted to yield the neuron’s phase preference. The same analysis applies to more complex spike patterns; coherence analysis of a model neuron that fires as a Poisson process with sinusoidal firing rate yielded similar results, although with lower coherence to the output pattern (Fig. 3, D–F).

The coherence analysis of experimental calcium imaging data from a single neuron (Fig. 3, G–I) exhibited similar features to those of the simulations. Over three ~50 s trials, fluorescence was measured from a ventral spinal interneuron and the flexor motor output recorded from the ipsilateral ventral root (1 trial shown in Fig. 3G). Both signals were visibly rhythmic, and the trial-averaged power spectra had significant peaks at 0.22 Hz (Fig. 3H). At this motor output peak frequency, the trial-averaged coherence magnitude was 0.85 (Fig. 3I), significantly above \( |C_{\theta}| = 0.05\) of 0.33, implying that the two signals were coupled at 0.22 Hz. The coupling between the two signals was also reflected by the stable coherence phase near 0.22 Hz, which became noisy at higher frequencies where the fluorescence and motor output were not coupled with any fixed timing relation. Data from three trials of the same neuron resulted in nearly identical coherence magnitudes and phases near the motor output peak frequency, showing that the calculation was robust and insensitive to trial-to-trial variability. The trial-averaged coherence phase was \( -0.70\), after correcting for the slow calcium decay dynamics offset estimated in the model neuron: \( 0.44 - 1.14 = -0.70\). The negative coherence phase lies within the quadrant of \([-\pi/2, 0]\), which indicates that this cell became active before the peak of the ipsilateral flexor motor activity.

We note that, in this analysis, zero coherence phase at the oscillating frequency indicates coupling of fluorescence and ipsilateral flexor motor activity with no delay. Therefore zero phase is approximately the time of the peak of the ipsilateral flexor ventral root burst activity (this would be exact if the oscillating frequency is the only frequency component present). In contrast, in conventional spinal cord analysis, zero phase typically refers to the initiation of the ipsilateral flexor motor burst. Therefore the two phase definitions are different by \( \pi/2\). For consistency, we will report the phase values as derived from the coherence analysis.

### Coherence analysis is robust and better than inferring spikes from calcium for rhythmic data

To support the validity of coherence analysis for calcium imaging data, we used further simulations to confirm that the
estimated coherence phase, including the offset, is nearly constant under a variety of conditions. The offset phase value is a function of the output period and the calcium dynamics time constant, which came directly from the experiment (Figs. 1B and 2E). One concern is that calcium buffering may vary for different cell types (Lee et al. 2000). In the ventral spinal cord, when we simulated the decay time constant to be 1 SD longer than our measured mean decay time (from 1.6 to 2.3 s), the offset increased from 1.14 to 1.25, a small difference relative to the phase period of 2\pi. This small difference is not surprising because the calcium transient was modeled as an exponential decay function, so changing the time constant only has a modest effect on the shape of the initial peak, where the weight of the function is concentrated. We also examined cases where the model neuron has more complex spike patterns. When the model neuron fires more than one spike per output cycle, coherence analysis should consistently report the phase of the median time of the spikes relative to the circuit output. Indeed, when we simulated burst trains with multiple spikes per burst, the estimated coherence phase was only slightly changed from the train of single spikes (Fig. 3A; coherence phase \( \theta = 1.14 \); period \( T = 0.23 \) s; spikes at \( t = 0 \)) to trains with seven evenly spaced spikes per burst (\( \theta = 1.11 \); \( T = 0.23 \) s; spikes at \( t = -3T/10, -T/5, -T/10, 0, T/10, T/5, 3T/10 \)) or seven asymmetrically spaced spike bursts (\( \theta = 1.31 \); \( T = 0.23 \) s; spikes at \( t = -3T/20, -T/10, -2T/20, 0, T/10, T/5, 3T/10 \)). Typical Poisson spike trains with a sinusoidal firing rate (Fig. 3, D–F) also led to similar coherence phase values, as described above. These simulations showed that coherence analysis is a robust method to quantify the rhythmicity and phase of individual neurons from calcium imaging data.

Alternatively, it is possible to infer spikes from the calcium signal and estimate rhythmicity and phase via circular statistics, a method more commonly used for electrophysiological data in motor physiology (Berkowitz and Stein 1994; Kjaerulff and Kiehn 1996). Spike inference can be done by a variety of methods: here we tested two of the simplest approaches, thresholding and template matching. Using simulated data with added white noise, we compared the ability of coherence and the two spike inference methods to estimate rhythmicity and phase with increasingly noisy signals (Fig. 4). By repeating the analysis 100 times, we obtained multiple estimates at each noise amplitude using the three analysis methods. There were a number of observations. First, coherence and the \( r \) value from circular statistics, although both bounded by 0 and 1, are different quantities, so direct comparison of the values is not possible. Most notably, the \( P = 0.05 \) value is a constant for coherence (gray line in Fig. 4A) but is variable for the Rayleigh test for circular statistics and is dependent on the number of inferred spikes. Second, although the absolute values are not meaningful, given a fixed \( P \) value of 0.05, we can compare the ability to identify the true positives, rhythmic fluorescence with added noise that was correctly classified, and to reject false positives, noise-only fluorescence classified as rhythmic (Fig. 4B). Using both criteria, coherence analysis performed better than inference using template matching, which in turn was much better than thresholding. The same order of performance holds for estimating the correct phase with minimal spread (Fig. 4C). An intuitive interpretation is that coherence analysis uses information from the entire recording to estimate rhythmicity and phase, whereas spike inference methods focus on the small fraction of the signal around the calcium signal peaks. Third, although theoretically \( r \) value and coherence magnitude have a lower bound of zero, the actual mean \( r \) value and coherence magnitude for the special case of noise-only fluorescence were nonzero (Fig. 4A, noise \( = \infty \)). This is because each simulation run consists of three trials each with a limited duration that contained only \( \sim 14 \) cycles; the estimated \( r \) values and coherence magnitudes clustered near zero with a heavy one-sided tail above zero, yielding a mean minimum value \( \sim 0–0.1 \). Coherence tends to be more biased for small sample size (Bokil et al. 2007; Maris et al. 2007). Experimentally, bias can be reduced by obtaining data that include a large number of cycles, so nonrhythmic fluorescence would have a mean \( r \) value and coherence magnitude closer to zero.

Phase preference of neuronal populations in mouse spinal cord

Applying coherence analysis to our large-scale calcium imaging data, we quantified the timing between the peak activity of a large number of individual spinal interneurons and

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**FIG. 4.** Coherence analysis of noisy simulated data and comparison with spike inference methods. White noise was added to the simulated fluorescence generated using parameters for Fig. 3A. The amplitude of the noise was varied from 0 to 5 times the height of a fluorescence peak because of a single spike, plus the special case where there is only noise and no signal (= symbol). At each noise amplitude, we used 3 methods, coherence analysis and spike inference based on either thresholding or template-matching, followed by circular statistics to estimate rhythmicity and phase. We repeated the analysis 100 times, each a trial average of 3 traces, to generate a mean and SD of the estimates. A: estimate of rhythmicity, measured as coherence magnitude or \( r \) value from circular statistics, as a function of noise amplitude. Because both coherence magnitude and \( r \) value are bounded by 0 and 1, they are plotted with the same y-axis. The \( |C| \) = 0.05 value is indicated by a gray line. B: for coherence, fluorescence was classified as rhythmic at \( P \) value of 0.05 if coherence magnitude \( > |C| = 0.05 \). For \( r \) value, fluorescence was classified as rhythmic at \( P \) value of 0.05 using the Rayleigh test. C: estimate of phase as a function of noise amplitude. All error bars show \( \pm SD \) to display the spread of the rhythmicity and phase estimate.
the peak motor output measured from the ventral root motoneuron bursts. The phase offset caused by slow calcium dynamics was subtracted. Each neuron was classified as coherent (mean estimate of coherence magnitude $> |C_{lp} = 0.05|$; colored green) or not coherent (mean estimate of coherence magnitude $< |C_{lp} = 0.05|$; colored white). As defined, coherent interneurons are rhythmic and track the ipsilateral motoneuron output with a consistent phase and therefore may be part of the locomotor CPG. Coherent neurons that fire “in phase” with the ipsilateral motor output would have a coherence phase value near zero, whereas “anti-phase” would have a coherence phase value near $\pi$. As expected, coherent neurons tended to have raw fluorescence traces that oscillate (Fig. 2E). However, not all neurons with noticeable fluorescence transients were coupled to the motor output with a fixed phase, and coherence analysis rejected those neurons and classified them as not coherent (e.g., cells 8 and 9 in Fig. 2E).

In lamina VIII of the ventromedial region of the lumbar spinal cord, which has been implicated as the location of the core of the hindlimb locomotor central pattern generator circuit, most interneurons were coherent with a preferred phase to the network output (70%, 366/524 cells, $n = 6$ spinal cords; Fig. 5A). Of the coherent interneurons, a large proportion of cells (87%, 317/366) were associated with the ipsilateral half of motor activity, with the majority (53%, 194/366) of cells located at $L_1$ and $L_2$ spinal segments active in the first quarter of locomotor period, immediately before the peak of ipsilateral flexor motor activity. Overall, the coherent interneurons were not uniformly distributed across the locomotor cycle and had a mean phase of $-0.3 \pm 0.1$ ($P < 10^{-5}$, Rayleigh test). To study whether this coupling between interneuron activity and locomotor phase was unique to the ventromedial region in lamina VIII, we also imaged the more dorsally located intermediate lamina VII. There, the majority of the interneurons were also coherent (62%, 358/580 cells, $n = 3$ spinal cords; Fig. 5B), with a mean phase of $+0.3 \pm 0.1$ ($P < 10^{-5}$, Rayleigh test). The mean phase of dorsally located intermediate lamina VII interneurons significantly lagged the ventromedial interneurons ($P < 10^{-5}$, Watson-Williams test). In particular, more of the coherent neurons in the intermediate lamina VII had peak activity after the peak of ipsilateral motor output (70%, 251/358 vs. 46%, 172/366 in the ventromedial region; Fig. 5C).

Cell type–specific subset and spatial distribution of phase-preferred neurons

In addition to the population ensemble, we examined the phase preference of two subsets of interneurons identified by transcription factor–linked fluorescent protein expression. These cell type–specific data have been described in our previous reports on the functions of particular classes of interneurons (Kwan et al. 2009; Zhong et al. 2010). Here the aim is to compare the cell type–specific data to the population ensemble, facilitated by the large-scale application of coherence analysis. The Hb9 interneurons show rhythmic activity and have been proposed to participate in the pacemaker kernel of the locomotor central pattern generator (Hinckley et al. 2005; Wilson et al. 2005). Using Hb9-GFP/lacZ mice, we identified Hb9 interneurons by looking for GFP fluorescence and then using whole mount X-gal staining to confirm lacZ expression. Cells that only had GFP fluorescence but no lacZ expression were eliminated from the analysis. We found that the majority of Hb9-GFP/lacZ interneurons were rhythmically active by coherence analysis (74%, 28/38). These coherent Hb9 interneurons had a mean phase preference in the ipsilateral half of the motor cycle but with peak activity after the peak motoneuron activity (mean phase $= +0.5 \pm 0.4$, $P < 10^{-4}$, Rayleigh test; Fig. 6A). Compared with the population ensemble of coherent ventromedial interneurons, the coherent Hb9-GFP/lacZ interneurons fired at a significantly delayed phase ($P < 0.001$, Watson-Williams test). This result agrees with our
previous report that Hb9 interneurons are phase delayed relative to motoneuron activity, although the current mean phase of +0.5 ± 0.4 is further delayed compared with the previously reported value of +0.19, after converting to radian units (Kwan et al. 2009). This discrepancy arises because our previous study compared the timing between the onset of calcium peaks and the onset of motor activity, so the phase was calculated using the times of the onset of spiking activity, i.e., the first action potential. In contrast, the current coherence analysis measures phase of the median time of spikes with respect to the peak of motoneuron activity during the cycle.

The Chx10 interneurons are ipsilaterally projecting excitatory interneurons that have been proposed to drive commissural interneurons to help coordinate left-right alternation during fictive locomotion (Crone et al. 2008, 2009). Recent electrophysiological studies of the Chx10-CFP interneurons have shown that only about one half of Chx10-CFP interneurons are rhythmically active during fictive locomotion (Dougherty and Kiehn 2010; Zhong et al. 2010). Our imaging data also showed a subset (60%, 54/90; Fig. 6B) of Chx10 interneurons that are highly coherent with the motor output, as well as other Chx10 interneurons with nearly zero coherence. The coherent Chx10 interneurons had a mean phase of −0.3 ± 0.3 (P < 10^{-5}, Rayleigh test), so they fire slightly before or at the peak of the ipsilateral motor burst. Earlier electrophysiological measurements (Dougherty and Kiehn 2010; Zhong et al. 2010) identified a subpopulation of the rhythmically active Chx10 interneurons that were active in phase with the contralateral motor burst. Although we identified some neurons active in this phase (i.e., around π in Fig. 6), it is much smaller than in our earlier study; it is not clear why this was so. Compared with our earlier attempt to use correlation as a metric for calculating the phase preference of a smaller dataset of these Chx10 interneurons (Zhong et al. 2010), this analysis is superior in two ways. First, the phase offset caused by slow calcium dynamics has been subtracted. Second, rhythmicity is now characterized by coherence magnitude, which can be evaluated for statistical significance.

Imaging also enabled us to study the local spatial distribution of neuronal activity along the mediolateral and rostrocaudal axes. The imaging field of view spanned ~100 × 100 μm and contained on average 23 ± 1 cells. To visualize the coherence phase and magnitude of dozens of interneurons, false colors were assigned to each cell in a hue-saturation-value format (coherence phase: 0–2π mapped to hue; coherence magnitude: 0 to 1 mapped to saturation and value). The false colors were overlaid onto the locations of the cells. Using this graphical representation, we did not see any obvious organization for interneurons with similar preferred phases (Fig. 7A). Scatter plots of the differences of coherence magnitude and phase of all cell pairs as a function of their cell-to-cell distance also showed no significant evidence for spatial clustering (slope of IC = 2 ± 2 × 10^{-4} /μm, slope of phase = 1 ± 1 × 10^{-3} rad/μm; both values not significantly different from 0; Fig. 7B). We note that the ~100 μm length scale, limited by the maximum field of view that can be monitored while maintaining a fast imaging frame rate, would be too fine to observe gradients of cell activation over multiple lumbar segments.
DISCUSSION

We described an efficient method using two-photon calcium imaging and multitaper spectral coherence analysis for measuring the phase relationship of many neurons in a rhythmic neural circuit. We applied this analysis to the mouse spinal cord and characterized the spatiotemporal dynamics of spinal interneurons during fictive locomotion.

Implications for the mouse hindlimb locomotor circuit

The majority of spinal interneurons in the upper lumbar segments are rhythmic and reach peak activity in the early ipsilateral flexor motor phase during fictive locomotion. Our survey of >1,000 cells generated this result, which was previously inferred from blind electrophysiological recordings in the rat spinal cord using tetrodes or patch electrodes (Kiehn et al. 1996; Tresch and Kiehn 1999). Therefore rhythmic activity is common in cells within the ventral spinal cord and is unlikely to be exclusive to only a few cell types. Our interpretation is either that most ventral interneurons are locomotor-related or that cells unrelated to locomotor pattern generation in vivo can be entrained and exhibit some rhythmicity during fictive locomotion elicited by bath application of neurotransmitters. It is known that the hindlimb locomotor circuit can be modulated to generate multiple motor patterns (Grillner 2003) so neurotransmitter application, as an artificial means of exciting virtually all spinal neurons to elicit the motor rhythm, may simultaneously activate neurons that are normally used for different motor tasks. Avoiding this conundrum would require more detailed comparisons between locomotor activity in vivo and the different methods for eliciting fictive locomotion in vitro (Kiehn and Kjaerulff 1996; Klein et al. 2010).

The fact that many interneurons are rhythmic is also relevant for interpreting the increasing number of studies reporting genetically identified cell types that have locomotor-related activity (Butt and Kiehn 2003; Butt et al. 2005; Dougherty and Kiehn 2010; Hinckley et al. 2005; Wilson et al. 2005, 2010; Zagoraiou et al. 2009; Zhong et al. 2010). Because rhythmic activity is not unique to a few cell types, it is more insightful to compare the relative phases of the locomotor-related activity of the different cell types, which is possible with large-scale imaging and coherence analysis. Our data show that, although ventromedial interneurons generally precede the more dorsally located interneurons, specific neuronal classes like the ventromedial Hb9 interneurons have a mean phase that lags the more dorsal Chx10 interneurons, therefore displaying the opposite trend from the cell population. Along with more information about synaptic connectivity, knowing the activation timing of more cell types will enable us to elucidate how the different interneurons interact during fictive locomotion and to identify the early drivers and late followers of the locomotor circuit.

We observed a temporal order of population activation of the ventromedial interneurons before the more dorsally located interneurons during a locomotor cycle. Moreover, the mean phase of the ventromedial interneurons is negative, so they show peak activity before the peak of motoneuron root activity. Because of their early activation, the ventromedial interneurons are likely to be involved in the initiation and generation of the rhythmic motor pattern. This argument supports lesion (Bracci et al. 1996; Kjaerulff and Kiehn 1996) and activity-dependent staining studies (Kjaerulff et al. 1994) that identified the ventromedial region near the central canal as the locus of the rhythm generator. Spatially, we observed neighboring interneurons with different firing preferences, suggesting a lack of local order in interneurons phasing, so physically adjacent neurons are not constrained to fire synchronously.

Large-scale approach for analyzing rhythmic neural circuits

We used coherence analysis to quantify multi-cell calcium imaging data to determine the phase of neuronal activity in a rhythmic neural circuit. We showed how this large-scale recording approach can also provide spatial and cell type information. Aside from the imaging approach, cell type–specific spike–oscillation phase relationships can be measured by single-unit electrophysiology, either optically targeted (Margrie et al. 2003) or followed by juxtacellular labeling (Klausberger et al. 2003; Pinault 1996). However, the yield is low and limited to one to several neurons per experiment. Ensemble recording using tetrodes or silicon array probes followed by optogenetic identification is also possible (Lima et al. 2009).

Although imaging facilitates large-scale recording, the temporal dynamics of calcium transients would make it challenging to study rhythms at higher frequencies. Imaging was well suited to the neonatal (P1–P4) mouse spinal cord because the period of fictive locomotion at this early age (~3–5 s) is longer than the calcium dynamics time scale (1.6 s). In a rhythmic circuit that operates at a higher frequency, the calcium peaks from each cycle would overlap and reduce the effective signal-to-noise ratio. The amount of overlap depends on the calcium decay time constant, which varies based on experimental conditions such as the intracellular dye concentration, temperature, and somatic calcium handling dynamics, which may differ for neurons in different brain regions. In areas such as the neocortex, where the decay time constant is in the order of hundreds of milliseconds (Garaschuk et al. 2006; Stosiek et al. 2003), the temporal precision should improve. One caveat with coherence analysis of calcium imaging data are that the phase is measured with respect to the peak activity, but the actual duration and exact pattern of spikes are not directly measured. Moreover, because calcium reflects suprathreshold activity, the subthreshold membrane potential and synaptic events are not known.

There are several advantages for using coherence analysis, instead of spike inference, to quantify rhythmicity in single-cell resolution calcium imaging data. First, although the analysis requires prior measurement of the calcium dynamics time constant to correct for the phase offset caused by the slow decay of the calcium signal, the calculations are straightforward and can be easily applied to a large dataset with few preconditions. The analysis skips the step of inferring spikes from the calcium signal, which requires parameter tuning. Second, the significance of coherence phase can be tested with well-established circular statistics methods (Zar 1999). Statistically analyses specific to coherence have also been developed for estimating errors (Mitra and Pesaran 1999) and testing hypotheses (Bokil et al. 2007; Maris et al. 2007). Third, noise could lead to error in spike inference including missed spikes, false positives, and displaced spike times, which together worsen the rhythmicity estimates. The relative contribution of these errors depends on the noise structure and algorithm parameters so we did not attempt a full theoretical comparison. Nonetheless, in our analysis of simulated data (Fig. 4), coher-
ence analysis was better than spike inference methods at identifying rhythmic fluorescence, rejecting noise, and giving the correct phase estimate. For spike inference, faster scanning and more advanced spike inference algorithms will improve the estimate, although detection fidelity will ultimately be limited by the calcium decay constant, which is relatively long for neurons with high firing rates. In those cases, it may be advantageous to deconvolve the calcium signal to obtain a continuous firing rate (Yaksi and Friedrich 2006) and apply circular statistics.

Several previous imaging studies have used a frequency-domain approach to increase the signal-to-noise ratio by using a fixed-frequency, periodic stimulus (Cacciatore et al. 1999; Gandhi et al. 2008; Kalatsky and Stryker 2003; Taylor et al. 2003). By stimulating and recording at one specified frequency, this lock-in scheme improves signal detection by rejecting the more broadband noise at other frequencies. Our in vitro experiments have a quiet baseline (Fig. 1), but for in vivo calcium signals contaminated with neuropil contributions, the lock-in signal detection is likely to be useful.

Coherence analysis can be improved using wavelet transform (Grinsted et al. 2004), which will better handle data with time-varying locomotor cycle frequency. For this analysis, the data consisted of multiple, ~50 s trials where the locomotor cycle frequency was relatively constant, and Fourier transform was sufficient. If longer, single-shot recordings (e.g., >10 min) are needed to examine the evolving locomotor cycle frequency, the nonstationary data would be more suited for wavelet transform and cross-wavelet coherence analysis (Mor and Lev-Tov 2007), although the choice of wavelet and statistical tests will have to be carefully considered (Torrence and Compo 1998). Furthermore, calcium imaging data analysis can benefit from automated region-of-interest detection of cell bodies. One recent algorithm uses the sparseness in temporal and spatial structures for image segmentation (Mukamel et al. 2009). Unfortunately, rhythmic circuits generate data where many cells share similar rhythmic activity, which is no longer temporally sparse (Kwan 2010). These circuits will therefore require alternate methods for automated cell identification.

The approach described here could be applied to other neural circuits that generate slow rhythmic outputs, such as the respiratory and masticatory networks. Central brain regions also exhibit oscillations in relatively low-frequency ranges such as slow wave sleep that can be studied with imaging. Distinct cell types are important for regulating rhythmic circuits, so large-scale imaging and analysis approaches will become essential for studying large, complex rhythm generator circuits.

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DISCLOSURES

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