Preferred locomotor phase of activity of lumbar interneurons during air-stepping in subchronic spinal cats

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AuYong N, Ollivier-Lanvin K, Lemay MA. Preferred locomotor phase of activity of lumbar interneurons during air-stepping in subchronic spinal cats. J Neurophysiol 105: 1011–1022, 2011. First published November 17, 2010; doi:10.1152/jn.00523.2010.—Spinal locomotor circuits are intrinsically capable of driving a variety of behaviors such as stepping, scratching, and swimming. Based on an observed rostrocaudal wave of activity in the motoneuronal firing during locomotor tasks, the traveling-wave hypothesis proposes that spinal interneuronal firing follows a similar rostrocaudal pattern of activation, suggesting the presence of spatially organized interneuronal modules within the spinal motor system. In this study, we examined if the spatial organization of the lumbar interneuronal activity patterns during locomotor activity in the adult mammalian spinal cord was consistent with a traveling-wave organizational scheme. The activity of spinal interneurons within the lumbar intermediate zone was examined during air-stepping in subchronic spinal cats. The preferred phase of interneuronal activity during a step cycle was determined using circular statistics. We found that the preferred phases of lumbar interneurons from both sides of the cord were evenly distributed over the entire step cycle with no indication of functional groupings. However, when units were subcategorized according to spinal hemisords, the preferred phases of units on each side largely fell around the period of extensor muscle activity on each side. In addition, there was no correlation between the preferred phases of units and their rostrocaudal locations along the spinal cord with preferred phases corresponding to both flexion and extension phases of the step cycle found at every rostrocaudal level of the cord. These results are consistent with the hypothesis that interneurons operate as part of a longitudinally distributed network rather than a rostrocaudally organized traveling-wave network.

modularity; traveling wave; spinal cord injury; motor primitives; central pattern generator

The spinal circuitry is intrinsically capable of driving a variety of locomotor behaviors (Grillner 1981; Rossignol 1996), but information regarding the population activation dynamics of its interneuronal constituents and their overall organization remains sparse, especially in the adult cord. Of interest is how networks of spinal interneurons coordinate and govern the many facets of locomotion (flexor-extensor alternation, interlimb coordination, etc.) and the shaping of patterned motor output (McCrea and Rybak 2008). Although the connectivity and activity of a number of last-order interneurons have been characterized during fictive locomotion (reviewed in Brownstone and Wilson 2008; Jankowska 2008, 2001; Kiehn 2006; Kiehn et al. 2008; McCrea and Rybak 2008), the activity patterns of interneurons of the intermediate layers shown to be active during locomotor behavior (Carr et al. 1995; Cina and Hochman 2000; Dai et al. 2005; Huang et al. 2000; Kjaerulff et al. 1994) and the correlation of this activity to locomotion remains largely unexplored especially in a moving, adult spinal preparation.

It also remains to be determined if the spinal locomotor network’s topology leads to a spatial organization of interneuronal activity dynamics. Tresch and Kiehn (1999) have reported that segmental interneuronal population activity in the isolated neonatal rat spinal cord occurs in phase with the corresponding ventral root activity, suggesting that interneurons are arranged in close proximity to their target motorpools. More recently, Cuellar and colleagues (2009) have presented evidence showing that interneurons within the deep dorsal horn and intermediate zone fire in a spatiotemporal pattern consistent with a traveling-wave mechanism of activation during fictive scratching. Studies examining the activation patterns of motoneuronal pools have proposed that mammalian locomotion is produced by a rostrocaudal oscillation of the motoneuronal pools’ activity (Bonnot et al. 2002; Ivanenko et al. 2006; Prochazka et al. 2002; Yakovenko et al. 2002). These findings suggest that the activation dynamics of spinal interneurons may be directly related to their rostrocaudal location within the locomotor network.

Additionally, studies on a variety of movement behaviors have shown that movements are composed of few muscle synergies (Cappellini et al. 2006; d’Avella and Bizzi 2005; d’Avella et al. 2003; Drew et al. 2008; Hart and Giszter 2004; Ivanenko et al. 2004; Kiehn et al. 2008; Kjaerulff et al. 1994) and the correlation of this activity to locomotion remains largely unexplored especially in a moving, adult spinal preparation.

MATERIALS AND METHODS

Surgical preparation. Five adult domestic short hair female cats (2.8–3.5 kg) were used in these experiments. All animal care and procedures were approved by the Institutional Animal Care and Use Committee of Drexel and were performed according to National Institutes of Health guidelines. The animals were spinalized 10–14 days before the multunit recording experiments. On the day of the terminal recording experiment, three procedures were performed:

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bipolar EMG recording electrodes implantation, spinal cord lamincotomy, and mid-collicular decerebration.

**Spinalization.** Animals were spinalized under aseptic conditions at the T11–T12 vertebral level 10–14 days before the terminal experiment (Boyce et al. 2007). Anesthesia was induced with ketamine HCl (25 mg/kg im) and maintained with isoflurane (1.5–3.0% in oxygen) given through an endotracheal tube. A laminectomy was performed with rongeurs creating an opening (~5-mm wide by ~10-mm long) at the T11–T12 vertebral junction. The dura was carefully opened, and the spinal cord was severed with microscissors between the T11 and T12 roots. The completeness of the transection was verified visually with the aid of an operating microscope. Postprocedural care of the spinal animals was as in Boyce et al. (2007).

**Multiunit recording procedure.** Following a recovery period of 10–14 days, interneuronal recordings were made during air-stepping (Giuliani and Smith 1985, 1987) over the course of a terminal experiment. The acute experimental protocol included the induction of anesthesia, implantation of bifilar EMG recording electrodes in both hindlimbs, a laminectomy to expose the spinal cord, decerebration, and the neuronal recording experiment. Anesthesia for the acute experiments was induced at the onset and maintained until the decerebration procedure was completed. Anesthesia was induced and maintained as for the spinalization procedure. Blood pressure, heart rate, end-tidal CO₂, arterial oxyhemoglobin saturation, respiratory rate, and core temperature were recorded and monitored every 15 min. Intravenous fluids were administered at a rate of 20 mL/h, and body temperature was maintained and monitored between 37 and 39°C.

**EMGs recording electrodes and implantation.** EMGs were recorded bilaterally from seven hindlimb muscles: two knee extensors [biceps femoris anterior (BFA) and vastus lateralis (VL)], one knee flexor [biceps femoris posterior (BFP)], two ankle extensors [gastrocnemius medialis (MG) and soleus (SL)], one ankle flexor [tibialis anterior (TA)], and one bifunctional muscle, sartorius anterior (SA), which acts as a hip flexor and a knee extensor. Muscles were implanted with bifilar electrodes constructed with insulated multi-stranded stainless steel wires (AS 633; Cooner Wire, Chatsworth, CA). The electrodes were implanted using a curved needle and secured onto the muscle fascia using silk sutures. The remaining length of each wire electrode was passed subcutaneously along the lateral aspect of the hindlimb, exiting posterior and slightly inferior to the anterior iliac crest. Incisions were closed with sutures or skin clips. Proper localization of the electrodes into the muscles of interest was verified via direct electrical stimulation of the muscle and postmortem dissection.

**Spinal laminectomy and decerebration.** Laminectomy was performed using rongeurs on vertebrae L3-L6 to expose the spinal cord. Before the laminectomy, and every 6 h after the initial dose, dexamethasone (2 mg/kg iv) was administered to minimize spinal swelling.

Following the laminectomy, the animal was transferred and secured to a spinal stereotaxic frame. Warm mineral oil was applied to the cord to prevent drying; the dura was opened, and the lumbar segments identified by examining the vertebral exit levels of the dorsal roots. A mid-collicular decerebration was performed, and the brain rostral to the transection removed. Dextran was administered if needed to maintain blood pressure, and anesthesia was discontinued once the decerebration was completed. Interneuronal recordings started at least 1 h postdecerebration. Clonidine (500 µg/kg iv) and on occasions naloxone (500 µg/kg iv) were administered with air-stepping, which was initiated via stimulation of the perineal area.

**Interneuronal recording.** Extracelluar interneuronal activity was recorded using a pair of multichannel electrode arrays, one inserted into each side (left and right of the midline) of the spinal cord. Each array consisted of four silicon shanks with four Pt/Ir/Au electrode contacts (4×4 3-mm 100-125-177; Neuronexus, Ann Arbor, MI) per shank. Electrodes were distributed 100 µm apart vertically on each shank with each shank spaced 125 µm apart horizontally. This arrangement yielded a 4 × 4 rectangular recording region with a height of 400 µm and a width of 500 µm. Electrode surface area was 177 µm². The arrays were mounted on a custom electrode holder driven by a manual linear micromanipulator (M-633; Physik Instrument, Germany). The most medial shanks of the two arrays were separated by a gap of 1.2–1.4 mm to straddle the cord’s midline but located on a single plane. The electrode holder and micromanipulator were mounted onto a stereotaxic arch that permitted rostrocaudal and mediolateral positioning of the electrode arrays.

The arrays were inserted into the spinal cord such that the electrode grid lay in the cross-sectional plane and was centered between the dorsal entry zones, unless small adjustments in the mediolateral positioning had to be made to avoid blood vessels. The tips of the shanks were inserted to a depth of 1,500–2,000 µm with the aid of an operating microscope. Given the spatial arrangements of the electrode grid and the established thickness of spinal laminae, the recording region largely encompassed laminae V, VI, and VII, together forming the intermediate zone of the spinal gray. This was verified using fluorescence staining of the electrode tracks in a number of animals (DiCarlo et al. 1996; Naselaris et al. 2005).

Neuronal activity was recorded over the rostrocaudal extent of the spinal cord in successive trials. Recordings begun at the top of the L3 dorsal roots and progressed caudally in 1- to 2-mm increments until the bottom of the L6 dorsal roots. During a trial, electrodes were inserted to the targeted depth. For each trial, neuronal and EMG activities were recorded while the animal was quiescent (5-s duration) and then during a period of air-stepping (~30 s) elicited by perineal stimulation (Fig. 1). Upon completion of a trial, the electrodes were retracted and advanced caudally by 1–2 mm. A number of control trials were conducted where neural activity was recorded during passive motion of the hindlimbs, cutaneous input to the leg, flexion withdrawals evoked by pinching between the toes, and perineal stimulation before the administration of clonidine. Neuronal activity was highly attenuated or absent in most channels during these control trials.

**Data acquisition system.** Both neuronal activity and EMGs were synchronously captured using custom Labview software running on a PC equipped with three 16-bit resolution data-acquisition cards (PCI-6251; National Instruments, Austin, TX) operating on a common clock source. Neuronal and EMG activities were sampled at 40 and 2 kHz, respectively, and stored for off-line analysis.

Neuronal activity voltages were acquired using two unity-gain headstages (HST/1625–18P-GR; Plexon, Dallas, TX) amplified 50× by two 16-channel bandpass (100 Hz-8 kHz) preamplifier boards (PBX2/32p-G50, Plexon). EMGs were amplified with a bank of differential amplifiers (model 1700; A-M Systems, Carlsborg, WA) and band-filtered (30 Hz-1 kHz) before being sampled.

**Neural data processing.** Single-unit spiking activity was extracted offline from stored voltage records using the following standard techniques implemented in Matlab (The Mathworks, Natick, MA). Raw voltage records from each channel were zero-phase digitally filtered with a bandpass elliptical filter (bandpass frequency range: 400 Hz-5 kHz; peak-to-peak ripple 0.1 dB, minimum stopband attenuation 40 dB). Spiking activity was identified with a threshold crossing method (thresholds: ±3 standard deviation from the filtered-waveform mean). Candidate spike waveforms identified by a positive threshold crossing were aligned to their maximum point while those identified by negative threshold crossing were aligned to their minimum, respectively, and stored for off-line analysis.

Spikes waveforms identified from each channel were first automatically sorted with a sorting algorithm based on a mixture of multivariate t-distributions (Shoham et al. 2003). The automatic sorting results were verified visually by inspecting cluster separation based on principal component projections, waveform shapes, and time-plot amplitude graphs of neural activity for each trial. Only well-isolated units with I <2% of total spikes having interspike intervals <1 ms,
contrast, phasic modulation of the spike rate is apparent for the duration of air-stepping.

For each right step cycle, five values were calculated: 1) extensor duration ratio, 2) flexor duration ratio, 3) flexor phase ratio, 4) cycle duration ratio, and 5) left onset lag. The extensor duration ratio was calculated by dividing the right hindlimb extensor burst duration by the left hindlimb extensor burst duration, whereas the flexor duration ratio was calculated by dividing the right hindlimb flexor burst duration by the left hindlimb flexor burst duration. These two ratios provide a measure of the symmetry in the burst durations of the right and left hindlimbs. The cycle duration ratio was calculated by dividing the right hindlimb flexor onset phase (relative to the right step cycle) by the left hindlimb flexor onset phase (relative to the left step cycle), and this ratio describes the symmetry in flexion onset time between the two hindlimbs. The cycle duration ratio was calculated by dividing the right hindlimb step cycle duration by the left hindlimb step cycle duration. The left onset lag was defined as the phase of onset of the left hindlimb extensor within a right step cycle. For symmetrical stepping with similar muscle activation timing in both legs, these ratios should be 1.0. Significance of deviation from unity for each of the ratios was evaluated using the t-test with alpha set at 0.05.

Analysis of single-unit activity. Circular statistics were used to assess the modulation patterns of single-unit activity during a step cycle. Only units with at least four spikes/sstep cycle for four steps were included in the analysis. For a given spike train, the relative phase (\( \varphi \)) of each spike occurrence with respect to the containing step cycle was determined with the following equations (as presented in Drew and Doucet 1991):

\[
\varphi = \frac{\Delta t}{T} = \frac{t_{\text{spike time}} - t_{\text{step start time}}}{t_{\text{step end time}} - t_{\text{step start time}}}
\]

where \( \varphi \) ranges from 0 to 1, with 0 and 1 corresponding to the beginning and end of the step cycle, respectively. A histogram of the relative phases was then constructed using 50 equally spaced bins between 0 and 1. The relative phase values of a histogram’s bins were converted to radians (\( \theta \)) by multiplying the normalized phase (between 0 and 1) by 2\( \pi \). Following this transformation, the histogram values represented magnitudes (\( \tau \)) of vectors pointing in 50 equally spaced directions (\( \theta \)) around a circle. For many units, the activity was most concentrated around a particular phase, or preferred phase. The preferred phase was calculated from the histogram using vector averaging. The magnitude (\( r \)) and direction (\( \Phi \)) of the mean vector,
generated by vector averaging these 50 vectors, provides a concise description of the concentration of activity within a step cycle and can be calculated using Eq. 1 and Eq. 2 below. The preferred phase of a unit’s activity (i.e., the phase around which activity is concentrated, again ranging from 0 to 1) is the mean vector angle (Φ) normalized by 2π.

\[
\theta = 2\pi \varphi \\
X = \sum_{i=1}^{50} n_i \cos(\theta_i) \\
Y = \sum_{i=1}^{50} n_i \sin(\theta_i) \\
r = \sqrt{X^2 + Y^2} \\
\Phi = \tan^{-1}\left(\frac{X}{Y}\right) \\
s = -2 \ln(r)
\]  

The value of \( r \) spans the range from 0 to 1 and takes on a value of 0 when the histogram magnitudes are uniform and 1 when only a single bin has a nonzero value. An \( r \) of 0 indicates there is no modulation in activity since there is an equal occurrence of spikes at all points within a step cycle. An \( r \) of 1 indicates narrow modulation since spikes only occur within a single phase bin. The angular deviation (s) provides a measure of dispersion of a unit’s activity.

Interpretation of the preferred phase as the center of activity concentration is only accurate when the histogram is unimodal (Drew 1993). To determine if a histogram was significantly unimodal, a simple test was developed. The histogram of the relative phases was first filtered with a five-point center-average filter. A threshold (\( Thr \)) was then defined according to Eq. 4

\[
Thr = f_{n_{\text{min}}} + 0.25(f_{n_{\text{max}}} - f_{n_{\text{min}}})
\]

where \( f_{n_{\text{min}}} \) is the bin value of the minimum of the filtered histogram, and \( f_{n_{\text{max}}} \) is the bin value of the maximum of the filtered histogram. Peaks within the histogram were then defined as contiguous bins within the histogram that cross this threshold. A significantly unimodal histogram contains only one peak, and the area of the peak accounts for at least 50% of the total area of the histogram. To account for wrapped peaks (i.e., peaks that begin at the end of the step cycle and end at the beginning), the areas of these disjointed peaks were summed and considered a single peak.

Significance of a unit’s phasic modulation was examined with two statistical tests. The Rayleigh test for directionality (Drew and Doucet 1991) was used to test for the significance of the preferred phase. Only units with \( P \) values < 0.01 were included in our analysis. A second test examined the time dependency of spike occurrence. This test involved the generation of a surrogate data set obtained by randomly permutating the interspike interval (ISI) from a unit’s spiking activity. One-thousand surrogate spike trains were generated to form a test data set. Since each surrogate spike train was generated by shuffling the ISIs, the distribution of the ISI remained identical, but the time of spike occurrence was altered. The mean and standard deviation of the phase histogram was constructed from the test data set and compared with the unit’s phase histogram. Units with bin values 3 standard deviation outside of the test data set’s mean phase histogram were considered to show significant phasic modulation and included for further analysis.

**Correlation between the preferred phases and rostrocaudal locations of units.** The correlation between the units’ preferred phases and their rostrocaudal locations was evaluated using a non-parametric test proposed by Mardia (1976) to evaluate correlation between a circular and a linear variable. The test is based on rank ordering the preferred phases and rostrocaudal locations based on preferred phases. To break the ties in rostrocaudal locations (multiple units recorded at the same rostrocaudal level), we added an evenly distributed small random number (within ±50 μm) to the rostrocaudal locations of the units and ran a series of 100 correlations for the ipsilateral units’ preferred phases to rostrocaudal locations. The correlation values were then compared with the critical value for the test (based on a Chi-square distribution with \( \alpha = 0.05 \), and 2 degrees of freedom, see Mardia 1976) to establish significance of the correlation between the rostrocaudal location of the units and their preferred phases.

**EMGs preferred phases.** EMG activity of each muscle for each step cycle was segmented into 20 equally sized bins in the range from 0 to 1. For each bin, the mean value of the rectified raw EMG signal was calculated. An average profile of EMG activity during a step cycle was produced by averaging all values for all bins of the same phase. Least-square fitting with a wrapped Gaussian function was used to identify the preferred phase of muscle activation. A wrapped Gaussian function is the circular analog to a standard one-dimensional Gaussian function except its amplitude is defined as a function of angles (Swindale 1998). Similar to the cosine function, the wrapped Gaussian can also slide around the 0–1 phase and is thus able to capture activity that spans from the end of one step cycle to the beginning of the next. Both the preferred phase (i.e., mean) and the tuning width (i.e., standard deviation) are estimated directly as the parameters of the wrapped Gaussian function. The goodness-of-fit was evaluated by the \( R^2 \) value where an \( R^2 \) value > 0.6 is considered a good fit.

**Spike train-EMG correlation.** The temporal correlation between single-unit and EMG activity was quantified with the Spearman cross-correlation coefficient. For each spike train, a spike density representation was generated by binning the spike train into 0.5-ms bins and then smoothed with a 100-ms Gaussian window filter. A Spearman cross-correlation coefficient was then calculated between the spike density waveform and corresponding EMG activity of the right and left BFP (a flexor muscle) and the right and left soleus (an extensor muscle) over all steps with at least four spikes/cycle. In one animal, the left sartorius anterior was used instead of left BFP due to low signal quality.

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**Fig. 2.** Fluorescent photomicrograph of a 25-μm-thick transverse spinal cord section taken at the caudal end of the L3 spinal segment in 1 animal. The electrode arrays were coated with DIO (Molecular Probes, Eugene OR) and inserted at 2,000-μm depth with the center of the two arrays aligned on the cord midline (the distance between the 2 electrodes array was −1,500 μm). Electrodes tracks can be seen extending to the dorsal surface of the cord with the fluorescence being strongest at the dorsal aspect. The depth of the most ventral signs of fluorescence and the medio-lateral positions of the tracts match the stereotaxic coordinates (the most ventral fluorescence for the array on the right was on an adjacent section). The yellow box indicates the assumed region covered by the electrode array on the left based on the distances between shanks and sites. Rexed laminae for the L3 segment are overlaid in gray and indicate that recording sites were indeed located within the deep intermediate horns (laminae V–VII).
RESULTS

Summary. We isolated 971 single units from recordings made over 127 sites in five subchronic spinal cats during bouts of locomotion. In each cat, the spinal cord was mapped from the most rostral L3 rootlets to the most caudal L6 rootlets (average distance between most caudal and rostral sites was 37.38 ± 6.01 mm, with 15–33 penetrations made within that rostrocaudal extent). The majority (57%) of the recordings were made at a depth of 2,000 μm with the remainder of the sites recorded being within 500 μm of that depth. Figure 2 shows an example of the fluorescence markings obtained in one animal. The regions of fluorescence matched the recorded stereotaxic electrode depths and locations for the penetrations tested. On average, 6.61 ± 5.4 well-isolated single units were found over the 32 recording sites at each rostrocaudal position. The mean amplitude of the units isolated was 216.53 μV with an average signal-to-noise ratio of 4.12 ± 2.04. The firing rate of 75% of the units exhibited phasic modulation during locomotion (Fig. 1), as defined by the Rayleigh test for directionality (P < 0.01) and also when tested against a shuffled ISI surrogate data set.

Locomotor symmetry between the left and right legs during air-stepping was quantified using four ratio values: the extensor duration ratio, the flexor duration ratio, the flexor phase ratio, and the cycle duration ratio. The average extensor and flexor ratio values in all cats were not significantly different than 1.0 (t-test, P > 0.05), and neither were the average flexor onset phase and cycle duration ratio (t-test, P > 0.05). The regularity of left-right alternation was quantified by the phase within a right step cycle at which the left extensor onset, termed the left-lag, took place. For all cats, the average left-lag was not significantly different than 0.5 (t-test, P > 0.05). Overall, these results indicate that stepping was symmetrical with the left and right legs operating on a 1:1 stepping cycle.

Interneuron firing activity is modulated during locomotor cycle, and peak of activity is distributed evenly throughout a step cycle for upper intermediate gray interneurons. The preferred phases of the units exhibiting phasic modulation relative to the left or right step cycle, as defined by the period between the onsets of left or right soleus activity, respectively, were estimated using circular statistics methods (see Fig. 3). Of the 730 units that showed modulation in firing rate during locomotor activity, 645 exhibited statistically significant directionality (Rayleigh test for directionality P < 0.01) with respect to the right step cycle. The preferred phases of these units along with their standard deviations are plotted in Fig. 4B, ranked in order from the lowest to the highest phase. The preferred phases were distributed throughout the locomotor cycle as reflected by the width of the preferred phase distribution histogram (Fig. 4A) and the even spread between vectors in the polar plot of the preferred phases of the units exhibiting statistically significant directionality (Fig. 4C). When the recording locations of each unit were considered (i.e., when units were separated based on which side of the cord they were recorded from), the preferred phases of neurons segregated based on which side of the spinal cord they were recorded from. Units recorded on the right hemicord had preferred phases localized toward the first and last third of the right step cycle, whereas neurons recorded from the left side had preferred phases localized within the middle third of the cycle (Fig. 4, A and D). This trend was seen in all five animals (see Fig. 5), although fewer single units were recorded in the right electrode array in two animals (Fig. 5, D and E). The pooled preferred phase of units from the left side of the cord was found to be significantly different from that of units from the right side (Watson’s non-parametric 2-sample U-squared test) with an average preferred phase of 0.54 ± 0.16 and 0.9 ± 0.21 for the left and right side units, respectively (Fig. 4D). Similar segregation and statistical results were found when preferred phase was measured relative to the left step cycle. In that case, 652 units exhibited statistically significant directionality (Rayleigh test for directionality P < 0.01) with respect to the left step cycle and similar segregation based on hemicord recorded from.

The analysis of firing activity patterns indicates that a significant number of interneurons in the upper intermediate
zone shows a single peak in firing activity, and that the peak firing of the interneurons is relatively evenly distributed throughout the gait cycle. These results matched well with older results (Orlovskii and Feldman 1972) in decerebrate cats locomoting on a treadmill with and without afferent feedback.

Muscle activity shows a limited number of preferred phases contrary to the interneuron activity. Since similar results in the distribution of preferred phases were obtained with the units referenced to the right or left step cycle, the relationship between the preferred phases of single units and locomotor activity was examined with respect to the ipsilateral step cycle only (i.e., unit recorded on the left(right) side of the spinal cord was examined with respect to left(right) step cycle). The average or mean phase of muscle activity represents the point within the ipsilateral step cycle where activity is centered and maximal. The distribution of mean preferred phases for the flexor or extensor muscle selected from each group (see Materials and Methods) showed a defined peak within a step cycle (Fig. 6A). Mean preferred phases showed significant directionality for each of the muscles studied (Rayleigh test for directionality $P < 0.01$). The average mean phases of the selected ipsilateral flexors and extensors were 0.79 (0.081 SD) and 0.19 (0.077 SD), respectively. The average mean phases of contralateral flexors and extensors were 0.29 (0.079 SD) and 0.70 (0.079 SD), respectively (Fig. 6B).

In addition, the average phases of muscle activation for all ipsilateral and contralateral flexors and extensors were determined for each hindlimb. The ranges of mean phases obtained for all muscles grouped by function and hindlimb (ipsi- or contralateral) are shown in Fig. 6C. Flexors and extensors from both sides grouped together as shown on Fig. 6C. The average Pearson’s correlation coefficient between binned flexors EMGs across all cats was 0.64 ± 0.17, whereas the correlation coefficient between binned extensors EMGs was 0.83 ± 0.16. The average correlation coefficient between binned flexors and extensors EMGs was 0.5 ± 0.16 with the majority of the correlation not being statistically significant. Furthermore, binned extensors and flexors EMGs clustered into two groups used Ward’s clustering method (PASW Statistics; SPSS, Chicago, IL).

Thus, we found that contrary to the preferred phase results for the interneuronal population, muscles showed groupings or modularity in preferred phases. Modularity in muscle activity has been reported for various types of movements in different species (Cheung et al. 2005; d’Avella and Bizzi 2005; d’Avella et al. 2003; Hart and Giszter 2004; Kargo and Giszter 2008; Krouchev et al. 2006; Overduin et al. 2008; Ting and Macpherson...
We investigated the activity of lumbar interneurons during air-stepping and characterized the units by their preferred phases of firing. We found that the preferred phases of lumbar interneurons were evenly distributed over a step cycle and...
segregated based on spinal hemicords with units firing mostly during the extensor phase of the ipsilateral side. In addition, units’ preferred phases were weakly correlated with flexor and extensor muscles activity. Most importantly, we found no correlation between the preferred phases of units and their rostrocaudal locations along the spinal cord.

These results support a number of hypotheses about the spatial organization of upper intermediate gray spinal interneurons. First, similarity in the phasic activation of spinal interneurons spread along the rostrocaudal extent of the cord suggests that these interneurons operate as part of a longitudinally distributed network rather than as localized modules firing in some form of sequence. Second, the segregation of preferred phases according to the period of extensor activity in the hemicord from which they were recorded suggests that the phasic modulation of interneuronal activity in the upper intermediate zone is mostly related to the extensor phase of activity in that hemicord, whereas flexor-related interneurons may be located more ventrally. Third, the lack of correlation between interneurons’ preferred phases and rostrocaudal location demonstrates that activity does not propagate rostrocaudally at the interneuronal level during locomotor activity. Together, these results suggest that the localization of interneurons within the rostrocaudal extent of the spinal cord weakly correlates with their peak of firing activity during the step cycle.

Laminar and segmental distribution of rhythmic activity within the spinal cord. We targeted upper intermediate zone interneurons because studies employing activity-dependent markers localized locomotor-related interneurons within that region (Dai et al. 2005; Kjaerulff et al. 1994). Previous studies

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**Fig. 6.** Preferred phases of muscle activity. **A:** example of the wrapped Gaussian fitting procedure used to determine the mean phase of muscle activity. Least square fitting was performed on the average EMG activity histogram profiles for 4 exemplar muscles, 1 flexor and 1 extensor muscle from each hindlimb. The mean phase of activity was determined relative to the step cycle of the hindlimb ipsilateral to the unit’s hemispheric location. **B:** polar representation of the average (black tick mark) and standard deviation (indicated by the angular width of the wedge) of mean phase of activity of a single flexor and extensor muscle from the ipsilateral and contralateral side with respect to the hemispheric location of the unit. The average mean phases of ipsilateral flexors and extensors were 0.79 (0.081 SD) and 0.19 (0.077 SD), respectively. The average mean phases of contralateral flexors and extensors were 0.29 (0.079 SD) and 0.70 (0.079 SD), respectively. **C:** preferred phases of muscle activity for all extensors and flexors across all cats. The dots indicate the individual preferred phases of the average binned EMG activity profile for each of the muscles recorded. The wedges indicate the range of preferred phases for each of the muscle groups (ipsi- and contralateral flexors and extensors). Flexors and extensors showed similar preferred phases of activity for both the ipsi- and contralateral hindlimbs.

**Fig. 7.** Scatter plots of the mean phases of 1 flexor and 1 extensor activity for the ipsilateral and contralateral hindlimbs vs. the preferred phases of ipsilateral units are plotted separately on the left. These individual scattered plots are combined to generate the summary plot on the right. Unity lines are also shown for reference. Muscle activity showed no correlation with neuronal preferred phases for any of the muscles studied as shown for the individual plots or the combined plot. Muscle color legend as in Fig. 6.
of single-unit activity within this region found that interneuronal activity was phasically modulated during locomotor behavior in a variety of mammalian locomotor preparations (Baev et al. 1979; Butt et al. 2002; Orlovskii and Feldman 1972; Raastad and Kiehn 2000; Tresch and Kiehn 1999; Viala et al. 1991). In addition to finding both flexion and extension-related interneurons, these studies reported that the phases of interneuronal activation were widely distributed over the locomotor cycle (Butt et al. 2002; Orlovskii and Feldman 1972). The results of the present study extend upon these findings by examining the distribution of preferred phases over the rostrocaudal extent of the lumbar cord. Orlovskii showed a significant proportion of ipsilateral flexor-related interneurons during mesencephalic locomotor region stimulation evoked locomotion located in the deep dorsal horn and mediolateral ventral horn of the L6-S1 segments (Orlovskii and Feldman 1972), whereas Shefchyk et al. (1990) only found flexor-related rhythmically active flexor at the 1.7- to 2.8-mm depth of the L4 segment. In contrast, we mostly obtained extensor-related interneurons for the L3-L6 segments, which is in agreement with findings in both the neonatal rat and cat (Butt et al. 2002; Huang et al. 2000) for ipsilateral extensor-related commissural interneurons. Some of our interneurons may be commissural interneurons, although in those studies the interneurons’ period of activity tended to match the period of extensor muscle activity, which was not a finding for the majority of the interneurons we recorded.

Propagation of rhythmic activity within the lumbar cord intermediate gray. A recent study (Cuellar et al. 2009) showed that the activity of lumbar interneurons, as measured by the cord dorsum potentials during fictive scratching in cats, exhibits traveling wave dynamics. The cord dorsum potential activity was most pronounced in the rostral cord during the flexor phase, propagating caudally with the extension phase. This rostrocaudal wave of activity was also reflected in the sequential activation of 18 interneurons in the 1.2- to 2.2-mm-depth range within the L6 segment. Motoneuronal pools in the neonatal mouse (O’Donovan et al. 2005), as well as in cats (Yakovenko et al. 2002) and humans (Ivanenko et al. 2006), also display a rostrocaudal dynamics of activation, although the switching between peaks of motoneuronal activity is in

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**Fig. 8.** Histograms of the coefficients of determination ($R^2$, i.e., squared Spearman correlations values) between each spike train and all of the corresponding ipsilateral or contralateral flexors or extensors activity ($P < 0.05$ for all correlation values shown). The coefficient of determination values between the vast majority of the units and any of the muscles studied were weak ($\leq 0.5$) with few units exhibiting large values ($>0.5$) for any muscle. The activity of 14 units was found to correspond well (coefficient of determination $>0.05$) with the activity of the ipsilateral extensor. These results indicate that very few interneurons were tightly tuned to the muscular activity of the flexors and extensors sampled in this study.

**Fig. 9.** A representative example of the single units’ preferred phases from 1 animal plotted against their rostrocaudal recording locations. The plot on the left shows the relationship when preferred phase is measured relative to the left step cycle, and the plot on the right is for when the phase is measured relative to the right step cycle. Preferred phases did not correlate with rostrocaudal localization for the units studied.
behaviors has been attributed to a premotor neural strategy where blocks of movement behaviors. Modularity in movement synergies has been suggested to serve as modular building in primates (Overduin et al. 2008). The limited set of muscle postural tasks (Torres-Oviedo et al. 2006), and hand grasping (Oviedo and Ting 2007), cat locomotion (Drew et al. 2008) and locomotion (Ivanenko et al. 2004) and postural tasks (Torres-Oviedo et al. 2003) and scratching (Hart and Giszter 2004), human the behavior. Behaviors studied include frog kicking (d’Avella et al. 2006). The hypothesis that interneurons should display similar sequential patterns of activation is based on a model where interneuronal organization would parallel the motor pools’ topological organization (Cheng et al. 2002; Tresch and Kiehn 1999). As such, the present results do not support a traveling wave mechanism for two reasons: 1) the spatial distribution of preferred phases does not reflect a rostrocaudal progression of activity from swing to stance, and 2) neurons with preferred phases falling within the swing and stance phase were colocalized within the same transverse section. The differences observed may be due to either the type of preparation used (fictive with intact descending systems but no afferent feedback vs. spinal with degenerated descending systems but with afferent feedback) or the behavior studied (fictive scratching vs. air-stepping) or both.

Lack of clustering in interneuronal firing patterns. Studies employing EMG decomposition approaches find that a variety of behaviors involve only a few muscle synergies. Synergies are defined as groups of muscles activated synchronously, with different synergies being employed during different phases of the behavior. Behaviors studied include frog kicking (d’Avella et al. 2003) and scratching (Hart and Giszter 2004), human locomotion (Ivanenko et al. 2004) and postural tasks (Torres-Oviedo and Ting 2007), cat locomotion (Drew et al. 2008) and postural tasks (Torres-Oviedo et al. 2006), and hand grasping in primates (Overduin et al. 2008). The limited set of muscle synergies has been suggested to serve as modular building blocks of movement behaviors. Modularity in movement behaviors has been attributed to a premotor neural strategy where the redundancy problem of the motor system is solved through the use of a few types of premotor drives or modules (Bizzi et al. 2008). While we found evidence for modularity in the muscle EMG activity patterns with clustering techniques, we found no evidence in the firing patterns of the interneurons studied using a similar clustering approach. The preferred phases from each hemicord spanned a distinct and different range in reference to the ipsilateral step cycle but were evenly distributed within each of those ranges with no signs of concentration around particular directions, or grouping of the activity patterns.

Other studies examining interneuronal phases of activity during scratching behaviors have observed similar even distributions in the preferred phases of interneuronal activity (Berkinblit et al. 1978; Stein and Daniels-McQueen 2002) but have concluded in favor of a modular organization for the interneurons studied using additional criterias to group the interneurons into modules. Berkinblit et al. (1978) observed an even distribution of the onset phase of activity for interneurons during scratch in cats, but parsed the interneurons into three groups using a combination of the onset and offset phases of the interneurons as dividing criteria. The groupings seem to correlate with other parameters of the interneurons such as firing patterns at rest, during latent scratching, etc., but the statistical significance of those correlations was not established. Stein and Daniels-McQueen (2002) present convincing evidence that even though the range of interneurons preferred phase span, the scratch cycle in turtles, the interneurons clearly divide into groups based on their behavior during various forms of scratching or during “deletions,” a phenomenon where flexors or extensors are silent while the antagonists are still rhythmically or tonically firing (McCrea 2001). This suggests that it may be feasible to cluster our interneurons into groups if appropriate additional behaviors were evaluated. Deletions, for example, sometimes occurred in our preparations, but in an effort to map the activity throughout the cord, we did not obtain sufficiently long episodes of deletions in one spinal location for us to statistically establish the variation in the firing of the interneurons during those periods. Although interneurons recorded in this study did not readily cluster into populations based on firing patterns, they may operate as part of functional neuronal assemblies that are spatially distributed and intertwined, with neurons potentially participating in multiple assemblies (Jankowska 2008), which would explain the lack of clustering at the neuronal firing level.

Proportion of units related to afferent feedback. Interneurons in the intermediate gray receive inputs from groups I and II afferents of major hindlimb muscles (quadriceps, iliofossos, triceps surae, sartorius) as well as cutaneous or joint afferents and descending systems inputs, although the more dorsal interneurons (laminae V–VI) show no group I afferent input (Edgley and Jankowska 1987; Harrison and Jankowska 1985a,b). Evidence suggests that these interneurons are activated on full limb extension and involved in determining the transition from stance to swing (Aggelopoulos et al. 1996). Clarke’s nucleus is also located within these dorsal laminae, and dorsal spino cerebellar neurons show a significant peak in activity during the locomotor extension phase (Bosco et al. 2006). Interestingly, recordings from quadriceps during locomotion (Loeb et al. 1985) show onset of activity in spindle being concurrent with muscle activity, not preceding it as does

Fig. 10. Binned (5-mm bin size) rostrocaudal distribution of the number of ipsilateral units with preferred phases in the 1st (<0.3), 2nd (>0.3 and <0.6), and 3rd (>0.6) portion of the ipsilateral step cycle. Interneurons located ipsilaterally largely fall within the 1st and last third of the ipsilateral step cycle for all 5 animals, whereas interneurons located contralaterally fall within the middle third of the step cycle.
our interneuronal activity. Furthermore, the afferent activity of a number of muscles peaks during the swing phase (Loeb and Duysens 1979; Prochazka and Gorassini 1998; Weber et al. 2007). In summary, if our responses were simply representative of sensory recordings, they should show a significant proportion of interneurons active during the ipsilateral flexion phase, which we lack. Some of the activity recorded is likely to be from afferent activity, but based on our control experiments recording at the same location during passive and flexion withdrawal movements, activity during these movements is approximately one-half of what it is during active (as measured by the number of channels showing spiking activity). These results would suggest that a fair proportion of units recorded are related to locomotor activity rather than sensory feedback, although our results likely represent a mix of the two interneuronal populations.

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REFERENCES


