Coding of Locomotor Phase in Populations of Neurons in Rostral and Caudal Segments of the Neonatal Rat Lumbar Spinal Cord

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Tresch, Matthew C. and Ole Kiehn. Coding of locomotor phase in populations of neurons in rostral and caudal segments of the neonatal rat lumbar spinal cord. J. Neurophysiol. 82: 3563–3574, 1999. Several experiments have demonstrated that rostral segments of the vertebrate lumbar spinal cord produce a rhythmic motor output more readily and of better quality than caudal segments. Here we examine how this rostrocaudal gradient of rhythmogenic capability is reflected in the spike activity of neurons in the rostral (L2) and caudal (L5) lumbar spinal cord of the neonatal rat. The spike activity of interneurons in the ventromedial cord, a region necessary for the production of locomotion, was recorded intracellularly with patch electrodes and extracellularly with tetrodes during pharmacologically induced locomotion. Both L2 and L5 neurons tended to be active in phase with their homologous ventral root. L5 neurons, however, had a wider distribution of their preferred phases of activity throughout the locomotor cycle than L2 neurons. The strength of modulation of the activity of individual L2 neurons was also larger than that of L5 neurons. These differences resulted in a stronger rhythmic signal from the L2 neuronal population than from the L5 population. These results demonstrate that the rhythmogenic capability of each spinal segment was reflected in the activity of interneurons located in the same segment. In addition to paralleling the rostrocaudal gradient of rhythmogenic capability, these results further suggest a colocalization of motoneurons and their associated interneurons involved in the production of locomotion.

INTRODUCTION

The search for the neuronal elements responsible for the production of behavior can be pursued at many levels. At the level of spike activity, many researchers have looked for correlations between the action potential output of neurons or neuronal populations and aspects of a particular behavior. For instance, this approach has been used to identify cortical neurons potentially involved in the control of voluntary movement (Fetz 1993; Georgopoulos et al. 1988; Kalaska and Crammond 1992). This rationale has also been used to identify spinal interneurons involved in the production of limb rhythmic behavior. Several different experiments, performed in cat (Arshavsky et al. 1984; Berkinblit et al. 1978; Gossard et al. 1994; Shefchyk et al. 1990) rabbit (Viala et al. 1991), rat (Kiehn et al. 1996; MacLean et al. 1995), turtle (Berkowitz and Stein 1994a,b), and the mudpuppy (Wheatley et al. 1994) have examined the action potential output of individual neurons during the production of locomotion or scratching. All of these experiments have found large numbers of neurons producing action potentials in relation to locomotion, with different neurons firing at different periods in the locomotor cycle. With few exceptions, however, this work has not examined the localization of this activity within different segments of the spinal cord, nor has it examined how various aspects of the locomotor output are reflected in the activity of the population of spinal interneurons. The present study examines these issues by quantifying the locomotor related spike activity of neurons in the in vitro neonatal rat spinal cord.

The application of certain drugs to the isolated neonatal rat spinal cord evokes a rhythmic motor output similar to the locomotor pattern produced in intact animals (Kiehn and Kjaerulff 1996). Lesion experiments suggest that the activity of neurons in the ventromedial regions of the lumbar spinal cord is necessary for the production of hindlimb locomotion (Kjaerulff and Kiehn 1996). Several studies have also demonstrated a difference in the capability of different spinal segments to produce a rhythmic motor output in the neonatal rat (Bracci et al. 1996; Cazalets et al. 1995; Cowley and Schmidt 1997; Kjaerulff and Kiehn 1996; Kremer and Lev-Toy 1997). Rostral spinal segments appear to produce a rhythmic motor output more readily and with a stronger modulation than caudal segments. We examine here how the differences between the rhythmogenic capabilities of different spinal segments are reflected in the action potential output of neurons located in the ventromedial regions of the rostral and caudal lumbar spinal cord.

As a means of sampling the activity of a large number of neurons, we have developed the use of tetrodes (Gray et al. 1995) for recording in the spinal cord. Because the use of tetrodes in the spinal cord is novel, we compare the results obtained by their use to the results obtained using more standard techniques of interneuron recording in the in vitro spinal cord.

Parts of these results have previously been published in abstract form (Tresch and Kiehn 1998a,b).

METHODS

Preparation

Eighteen neonatal rats (1–4 days old) were used in these experiments. Methods for the isolation of the neonatal rat spinal cord in vitro have been described previously (Kiehn et al. 1996). Briefly, rats were anesthetized with ether, decapitated, and eviscerated. The spinal cord was exposed by removing the ventral vertebral bodies under cold, low (10%) calcium Ringer solution. The preparation was transferred to a recording chamber, pinned down with the ventral side up, and superfused with oxygenated (95% O2, 5% CO2) Ringer solution of the following composition (in mM): 128 NaCl, 4.7 KCl, 25 NaHCO3, 1.2 KH2PO4, 1.25 MgSO4, 2.5 CaCl2, and 20 glucose (pH 7.4) at room temperature. To allow intracellular and extracellular electrodes to be

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lowered into the cord, a small slit was made in the ventral surface of the cord approximately one-third of the distance from the midline to the lateral border of the spinal cord.

Induction and monitoring of locomotion

Rhythmic activity was induced by adding a combination of N-methyl-D-aspartate (NMDA; 1–7 μM, usually 6) and serotonin (5-HT; 1–10 μM, usually 6) to the perfusion medium. Ventral roots L2 and L5 from one side of the cord were placed in suction electrodes to monitor the rhythmic activity induced by the drug application. The activity of motoneurons in these roots alternates and corresponds to the activation of flexor (L2) and extensor (L5) muscles during 5-HT and NMDA-induced rhythmicity (Kiehn and Kjaerulff 1996). For the rest of the paper we will refer to this activity as locomotion. Root activity was amplified (10,000–20,000 times) and band-pass filtered (100 Hz to 1 kHz) before being recorded by Axon Acquisition software (Axoscope, Axon Instruments, Foster City, CA; sampling, 1 or 5 kHz) for later off-line analysis. Only those bouts of locomotion consisting of regular and well-defined alternation between L2 and L5 ventral roots were used for further analyses.

Intracellular recordings

All recordings, both intracellular and extracellular (see Extracellular recordings), were made in the ventromedial part of the spinal cord 100–350 μm from the ventral surface (corresponding to the ventromedial portions of lamina VII, lamina VIII, and lamina X) ipsilateral to the recorded ventral roots. Tight seal whole cell recordings (Kiehn et al. 1996; Raastad et al. 1996) were made in current-clamp mode using an Axopatch 1-D amplifier (Axon Instruments). Neurons were recorded without injection of bias current. The microelectrodes were pulled from thick-walled borosilicate glass without filaments to a final resistance of 5–10 MΩ. The pipette solution contained in mM: 138 K-glucuronate, 10 HEPES, 4 mM NaCl, 0.0001 CaCl2, 5 ATP-Mg, and 0.3 GTP-Li. The pH was adjusted to 7.3 with KOH. Only neurons that were not motoneurons were included in the present study. These neurons, and those recorded from extracellularly (see Extracellular recordings), are referred to throughout this study as “interneurons” to differentiate them from motoneurons. Although the term “interneuron” is often used to refer to spinal neurons only with local segmental projections (Jankowska 1992) as opposed to those with long-range propriospinal projections, ascending axons, or commissural projections, we will use the term “interneuron” indiscriminately because we have not identified the projections of the recorded neurons in this study. Interneurons were differentiated from motoneurons by not having an orthodromic spike in the nearby ventral root after inducing action potentials in the recorded neuron. The intracellular signal was collected on Axoscope together with the root recordings.

Extracellular recordings

Tetrodes (Gray et al. 1995) were used to record the extracellular activity of neurons. We followed the procedures described previously for the construction and use of tetrodes in other systems (Gray et al. 1995). A tetrode was constructed from four fine wires (nichrome; 0.0005 in. diam, HP Reid, Florida) insulated (parylene) except at the tip. The four wires were twisted together, and their insulation melted under mild heat to fuse them together. The exposed tips were then gold plated to obtain a final impedance of 100–700 kΩ. The opposite end of each wire was attached to a pin connector using silver epoxy. All signals were recorded single-ended with a common bath ground as reference, amplified (10,000–20,000) and filtered (100 Hz to 1 kHz) before being collected using software provided by DataWaves (DataWave Technologies, CO; sampling 20 kHz each channel). Candidate action potentials were identified by a simple threshold crossing on any of the four channels. When such an event was detected, the voltage recorded on all four electrodes for a period of 1 ms before and 3 ms after the threshold crossing was saved for off-line analysis. The collection of action potential data was synchronized with the collection of ventral root activity by an external clock fed to both data collection systems.

We identified off-line the action potentials of neurons from the recorded signals using custom software written in Matlab (MathWorks). Because the different tips of a tetrode will be located at different distances from a given neuron, the action potential of the neuron will appear with a different shape and amplitude on each channel. These differences define a distinct profile across the four channels for the action potential of a neuron, facilitating its identification and making it possible to distinguish from the action potentials of other neurons. For instance, the relative peak voltages recorded on two of the channels of the tetrode might distinguish between the action potentials of different neurons. An example of this analysis is shown in Fig. 1. Figure 1A shows the activity recorded on the four wires of a tetrode placed in ventromedial L5 during locomotion. The figure shows action potentials of several different neurons that can be distinguished by their relative amplitudes on each of the four channels. Figure 1B plots the amplitudes recorded on two of the channels of the tetrode shown in Fig. 1A. In this figure, several distinct clusters of points can be seen, each cluster reflecting the action potentials of different neurons with slightly different amplitudes on the two channels. We separated clusters from one another by placing ellipses or boxes around each cluster (not shown). The action potentials on each of the four channels corresponding to the different clusters identified in Fig. 1B are shown in Fig. 1C. The cluster labeled 0 was clearly cutoff at low amplitudes on channel 1 (as seen in Fig. 1B) and was therefore excluded from analysis. The clusters labeled 1 and 2, although apparently well separated from other action potentials, were excluded because there were too few spikes observed for them to perform spike-triggered averaging with the ventral root (see next paragraph). The remaining five clusters of action potentials were used in subsequent analyses. Clusters 5 and 6, although not separated from one another in the plot shown in Fig. 1B, were separable in the plot of a different set of parameters. Figure 1B also makes it clear that, although most action potentials could be assigned to one of the clusters, there were action potentials recorded that were not readily identifiable. Using such analyses we typically were able to discriminate between the action potentials of 1–3 simultaneously recorded neurons (at most 6) with one tetrode. We did not attempt to resolve superpositions of the action potentials of different neurons in these analyses. However, because of the low firing rate of neurons in this preparation, the contribution of such superpositions would be expected to be relatively minor.

To differentiate interneurons from motoneurons, we performed spike-triggered averaging of the nearby ventral root recording. For this analysis we included only neurons from which we recorded at least 50 spikes to obtain a reliable estimate of the average. Cells that had a well-defined action potential in the ventral root after the spike-triggered averaging were classified as motoneurons (~5–10% of the recorded neurons) and discarded from the study. This procedure also excluded those neurons from which we recorded <50 action potentials, such as clusters 1 and 2 in Fig. 1B.

Data analyses

PROCESSING OF VENTRAL ROOT ACTIVITY AND ANALYSIS OF THE LOCOMOTOR CYCLE. The ventral root activity was zeroed, rectified, and filtered (1–10 Hz) using a digital filter implemented in Matlab. This filtered signal was then normalized so that it varied between −1 at its minimum and +1 at its maximum. An example of the rectified L2 and L5 ventral root activity evoked during locomotion,
along with the filtered root activity is shown in Fig. 2A. We then defined the locomotor cycle with reference to the activity in both of these roots (dual reference) (Berkowitz and Stein 1994a,b). We defined the transition from the activation of L2 to the activation of L5 as the time at which the filtered L5 recording became larger than the filtered L2 recording. Similarly, we defined the transition from activation of L5 to activation of L2 as the time at which the filtered L2 recording became larger than the filtered L5 recording. An example of the divisions of locomotion produced by this method is shown in Fig. 2A. This division of the locomotor cycle corresponded very well to that suggested by visual inspection. The division of locomotion was always confirmed visually, and only periods of stable locomotion were used for further analysis. As can be observed in Fig. 2A, the relative durations of the L2 and L5 bursts were not always the same, either within the same bout of locomotion (as shown in Fig. 2A) or between different bouts. To eliminate these differences, we normalized each portion of the locomotor cycle separately. The transition from L2 to L5 bursts was always used to define the middle of the cycle, and the preceding L2 burst and the following L5 burst were each rescaled to take up half the cycle. The locomotor phase was then expressed in terms of angular coordinates (Berkowitz and Stein 1994a,b; Drew and Doucet 1991; Kjaerulff and Kiehn 1996): the first half of the cycle with L2 most active always lasted from 0 to 180°, and the second half of the cycle with L5 most active always lasted from 180 to 360°. This expression of the locomotor cycle into phase angles allowed us to apply circular statistical analyses to the recorded data.
(see Expression of Neural Activity Relative to Locomotion). An example of the rescaling is shown in Fig. 2B. Here the filtered activity of the L2 and L5 ventral roots is shown as a function of the normalized locomotor phase. Note that despite the variations in burst durations seen in Fig. 2A, the time course of each ventral root burst is very similar after rescaling. The depth of modulation of the activity in each ventral root was also calculated as has been described previously as the difference between the maximum and minimum activity level of the root divided by the maximum level (“relative modulation amplitude”) (Kjaerulff and Kiehn 1996). This calculation was performed on the filtered, but nonnormalized, ventral root recordings.

Expression of Neural Activity Relative to Locomotion. The locomotor phase was divided into 10 bins: 5 in flexion and 5 in extension. The total number of action potentials produced by a neuron within each bin for a given cycle was divided by the amount of time spent within that bin. This value is the firing frequency of the neuron for that phase bin across all locomotor cycles. This process was repeated for each phase bin. An example of such a locomotor phase-related histogram is shown in Fig. 2C. This histogram was derived from the activity of the intracellularly recorded neuron shown in Fig. 2A. The error bars in this figure reflect the variability (1 SD) in the firing frequencies recorded between locomotor cycles.

We assessed whether the activity of a neuron was modulated by the locomotor cycle using the mean resultant length (R value) of each neuron’s phase histogram (Batschelet 1981). The R value indicates how well a circular distribution is described as unimodal and has been used previously to assess the modulation strength of neuronal spike discharge (Berkowitz and Stein 1994b; Drew and Doucet 1991; Westberg et al. 1998). A neuron that fires only in one phase bin will have an R value of 1, whereas a neuron that fires equally in each phase of locomotion or that is symmetrically multimodal will have an R value of 0. The significance of the R value was assessed by the Rayleigh test (Batschelet 1981). The number of observations (N) for Rayleigh’s test was chosen to be the total number of spikes recorded from each cell (Berkowitz and Stein 1994b; Drew and Doucet 1991). The activity of a neuron was considered to be locomotor related if its phase histogram had a significant R value.

One relevant aspect of the locomotor-related histogram for a neuron is the part of the locomotor cycle in which the neuron is most active. This aspect was calculated as the mean phase angle (Batschelet 1981). Another aspect that we assessed was the strength of modulation of each individual neuron. This modulation can be calculated as the mean phase angle (Batschelet 1981).

FIG. 2. Expression of ventral root activity and neuronal activity in angular coordinates. A: activity of a neuron recorded intracellularly with tight-seal whole cell methods in ventromedial L5 during locomotion induced by a combination of 5-HT and NMDA (6 μM and 6 μM, respectively). The rectified activity of L2 and L5 ventral roots is shown below (2nd and 3rd traces) along with the filtered and amplitude-normalized version of this activity (4th and 5th traces). Bottom trace: division of the locomotor cycle into L2 (indicated when the trace is high) and L5 bursts (indicated when the trace is low). One locomotor cycle consisted of one L2 and one L5 burst. The relative durations of each of the ventral root bursts were rescaled so that each locomotor cycle was made up of equal portions L2 and L5 bursts (B). This rescaling ensured that the L2 ventral root burst always lasted from 0 to 180°, and the L5 ventral root burst lasted from 180 to 360°. The firing rate of individual neurons was also expressed in angular coordinates as shown in C, in which the mean and standard deviation of the firing rate of the neuron in A for each of 10 bins of locomotor phase, 5 bins in flexion and 5 in extension, is plotted.
RESULTS

Characteristics of drug-induced locomotor activity recorded in the ventral roots

As has been observed previously, the combination of NMDA and 5-HT bath applied to the neonatal rat spinal cord evoked stable long-lasting alternating bursts of activity in L2 and L5 ventral roots on the same side of the spinal cord (Sqalli-Houssaini et al. 1993). This rhythmic alternation between L2 and L5 ventral root activity reflects the activation of flexor and extensor muscles during the production of locomotion (Izuka et al. 1997; Kiehn and Kjaerulff 1996). With the drug concentrations used in this study, the mean duration of each locomotor cycle was 1.97 ± 0.59 (mean ± SD) s, composed of 0.99 ± 0.29 s of L2 and 0.98 ± 0.29 s of L5 periods. Each bout of locomotion consisted of an average of 65 ± 29 locomotor cycles (minimum of 10).

We also calculated the relative modulation amplitude (Kjaerulff and Kiehn 1996) or depth of modulation of both the L2 and L5 ventral roots. The average depth of modulation of L2 was 0.42 ± 0.16, whereas the depth of modulation of L5 was 0.32 ± 0.14. The depth of modulation of the L2 ventral root was significantly larger than that of the L5 ventral root (P < 0.01).

Neuronal database

A total of 153 interneurons was recorded from the ventromedial regions of L2 (n = 65) and L5 (n = 88) in these experiments. Of these, 49 L2 and 63 L5 neurons were recorded extracellularly, and 16 L2 and 25 L5 neurons were recorded intracellularly.

Similar proportions of neurons in each segment were significantly related to the locomotor cycle. Thus in L2, 72% (41/65) of neurons were significantly related to the locomotor cycle, whereas in L5, 77% (61/88) of neurons were related to the locomotor cycle. The percentage of rhythmically active cells reported here is comparable to the percentages obtained with tight-seal whole cell (Raastad et al. 1996) and cell-attached (Raastad and Kiehn 1999) recordings. In all subsequent analyses, we examine only those neurons that were significantly related to the locomotor cycle.

Phase relationships of the L2 and L5 interneuron populations

Figure 3 shows some examples of the relationship between the spike activity of individual spinal interneurons and the locomotor phase. The spike activity is expressed as the average firing frequency in each of 10 bins: 5 during the L2 burst and 5 during the L5 burst (see METHODS). In Fig. 3, these 10 values are plotted at angles around a circle representing the locomotor cycle. The locomotor cycle begins at 0° at the right and advances clockwise, through the L2 burst on the bottom of the circle (0–180°), then through the L5 burst on the top of the circle (180–360°). The superimposed curved lines show wrapped normal distributions fit to each of these tuning curves. Figure 3A shows two neurons firing during the L2 ventral root burst, one recorded in the L2 spinal segment and the other recorded in L5. Figure 3B shows two neurons firing during the transition from the L1 burst to the L5 burst. Figure 3, C and D, shows neurons firing during the L5 burst and at the transition from the L5 to the L2 burst. We show these histograms to demonstrate that neurons in both L2 and L5 were active in different portions of the locomotor cycle.

As a simple means of summarizing the interneuronal activity in L2 and L5, we examined the averaged activation of locomotor-related neurons in each segment. We first eliminated differences between overall firing rates of different neurons by scaling the maximum of each phase histogram to one. We then simply averaged together the activity of all neurons within a given segment, separately for each of the 10 locomotor phases. The resulting averages are shown in Fig. 4. As can be seen in the figure, the averaged activity of the population of L2 neurons is maximal during the phase of locomotion in which the L2 ventral root is active (0–180°) and minimal when the L5 ventral root is active (180–360°). Conversely, the averaged activity of the population of L5 neurons is maximal when L5 is active and minimal when L2 is active. These patterns indicate that L2 and L5 neural populations tend to be active in phase with their homologous ventral root. However, this tendency appeared to be stronger for L2 neurons than for L5 neurons: the difference between the maximal and minimal activity in L2 was larger than that of L5 (0.36 vs. 0.16).

Figure 5 shows a different summary of the locomotor-related histograms of L2 and L5 interneurons. In this figure, the phase histogram of each neuron was again normalized so that the activity in its maximum phase has the value one. The activity of each neuron was then coded so that a normalized activity of one was represented by a red square, an activity of 0 was represented by a blue square, and intermediate levels of activity were represented by intermediate colors as indicated by the scale bar. Each row represents the activity of one neuron at each of the 10 bins of locomotor phase. The neurons in each segment were then sorted according to the phase of locomotion in which they were maximally activated. This analysis shows that the peak activity of neurons in both L2 and L5 was distributed throughout the locomotor cycle. Two differences between the L2 and L5 neuronal populations are clear, however. First, although the majority of L2 neurons was maximally active when the L2 ventral root was most active (0–180), the majority of L5 neurons was maximally active when the L5 ventral root was most active (180–360°). However, this tendency appeared to be stronger for L2 neurons than for L5 neurons: the difference between the maximal and minimal activity in L2 was larger than that of L5 (0.36 vs. 0.16). Second, the activity of L2 neurons appeared to be more strongly modulated by the locomotor cycle than the activity of L5 neurons. This stronger modulation of L2 neurons is reflected in Fig. 5 by the higher proportion of blue squares in the L2 illustration than in the L5 illustration. This difference suggests that the spike output of L2 neurons was more broadly related to the locomotor cycle than that of L5 neurons.

Both of these observations, of a weaker modulation of L5 neurons and of a broader distribution of L5 neuronal activity, suggest that the rhythmic signal of the L2 neuronal population was stronger than that of the L5 neuronal population. These differences would both be expected to contribute to the lower modulation of the averaged L5 population activity shown in Fig. 4, and they parallel the rostrocaudal gradient of rhythmicogenic capability described in the INTRODUCTION. We now examine more systematically these differences in the distributions of preferred locomotor phases and in the modulation of activation of L2 and L5 neural populations.
Preferred phases of activity for L2 and L5 neurons are different

The results shown in Fig. 5 suggested a difference in the distributions of activity in L2 and L5 through the locomotor cycle. To examine this difference further, we compared the distributions of mean phases of L2 and L5 neurons. The mean phase of a neuron is the phase of locomotion in which a neuron is most active. Figure 6 shows the numbers of neurons in L2 and in L5 with mean phases in each of the different phase bins. The distributions of mean phases for both L2 and L5 neurons were significantly different from a uniform circular distribution ($P < 0.01$ for both) and from each other ($P < 0.01$). Thus the majority of L2 neurons (32/41) had their mean phase in the first half (0–180) of the locomotor cycle, whereas the majority of L5 neurons (39/61) had their mean phase in the second half (180–360) of the locomotor cycle. The proportion of L2 neurons in the first half of the cycle was larger than the proportion of L5 neurons in the second half (78 vs. 64%). These differences are summarized in the histograms to the right, which have pooled L2 and L5 neurons with mean phases in either the first or second half of the locomotor cycle. These findings demonstrate that the neuronal populations in L2 and L5 tend to

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**Fig. 3.** Examples of the locomotor-related activity of neurons in L2 and L5 lumbar segments. The mean activity of neurons in each of 10 phase bins of locomotion was plotted around a circle. A–D: activity in neurons recorded in either L2 (left column) or L5 (right column). In these examples the mean phase of activity was in the middle of the L2 burst (A), at the transition from the L2 to L5 burst (B), in the middle of the L5 burst (C), and at the transition from the L4 to the L2 burst (D). Circle in the bottom right shows the progression of a locomotor cycle in this angular representation. Curved line overlaying the plot for each cell shows a wrapped normal distribution fit to the phase histogram (see METHODS).
be active in different portions of the locomotor cycle. For both populations, neurons tended to be active in phase with the ventral root of the segment in which they were located.

**L₂ neurons are modulated less during locomotion than are L₅ neurons**

In addition to the differences in distributions of preferred activity, the results presented in Fig. 5 also suggest that, in general, L₂ neurons have a stronger modulation of firing as compared with L₅ neurons. We examined this apparent difference in modulation strength in more detail by comparing different characteristics of the firing of L₂ and L₅ neurons. As described in METHODS, the modulation strength of a neuron is determined both by the depth of its modulation and by the sharpness of its modulation. Both of these parameters differed between neurons in L₂ and L₅ (P<0.05). The depth of modulation of L₂ neurons was 0.85±0.16, whereas that of L₅ neurons was 0.74±0.24. The average width of modulation of L₂ neurons was 0.47±0.17 (47% of the locomotor cycle); that of L₅ neurons, 0.56±0.22 (56% of the locomotor cycle). Thus L₂ neurons were both activated stronger and in a more restricted portion of the locomotor cycle than L₅ neurons.

We further investigated these points by comparing the R values for L₂ and L₅ neurons (Fig. 7). The R value combines information about both the width and depth of modulation and gives an estimate of the concentration of spikes in the locomotor cycle. The mean R value for L₂ neurons (0.45±0.21) was significantly larger (P<0.01) than the mean R value for L₅ neurons (0.35±0.21). Again, these findings suggest that L₅ neurons were not as strongly modulated during locomotion as L₂ neurons.

**Possible mechanisms for the difference in modulation strength**

There are several potential explanations for the difference in modulation strength between L₂ and L₅ neuronal populations. One possible cause of this difference could be a difference in the overall level of activity of neurons in L₂ and L₅. As can be seen in Fig. 8A, neurons with a higher rate of activity tend to have lower R values (see also Berkowitz and Stein 1994b). The average firing frequency over the entire locomotor cycle was...
significantly larger for L5 neurons than for L2 neurons (2.57 ± 2.05 vs. 2.09 ± 1.74, \( P < 0.01 \)). The difference in \( R \) values shown in Fig. 7 might therefore have been due to this difference in firing rate between the two neural populations. Figure 8A shows, however, that L2 neurons have higher \( R \) values than L4 neurons across different levels of mean firing rate. This result suggests that differences in mean firing rate between the two segments was not the only contributing factor to the difference in \( R \) values.

Another possible cause of the difference between L2 and L5 neurons is that the modulation of spike activity of a neuron might be dependent on the locomotor phase in which it fires. For instance, neurons that were active in the flexor cycle might in general have higher \( R \) values than neurons active in the extensor cycle. Such a difference, combined with the differential distribution of mean phases shown in Fig. 6, might result in the generally lower \( R \) values of L5 neurons. We therefore examined the dependency of \( R \) values on the locomotor phase. We examined this dependency only for L5 neurons because in this study we did not observe L2 neurons with mean phases of activity in all phases of the locomotor cycle (note the gap of L2 neuron mean phases in Fig. 6). As seen in Fig. 8B, there was no difference between the average of \( R \) values for L5 neurons in the first and second halves of the cycle (0.37 vs. 0.37). This observation suggests that the depth of modulation of a neuron was not primarily determined by the phase of locomotion in which it was active.

We have interpreted these results in relation to the rostrocaudal gradient of rhythmogenic capability of the spinal cord. However, because we have compared the properties of interneurons in only two segments, it is not clear whether these results would generalize to interneurons in other rostrally and caudally located segments. We therefore performed additional experiments examining the activity of interneurons in the L3 and L6 spinal segments. We recorded the locomotor related activity of 9 neurons in L3 and 39 neurons in L6. The mean \( R \) value of locomotor related neurons in L3 was 0.64, whereas the mean \( R \) value of locomotor related neurons in L6 was 0.19. These results, although from a limited data set, are consistent with the conclusion that the modulation strength of interneurons in rostral spinal segments is stronger than that of interneurons in caudal spinal segments.
Comparison of extracellular and intracellular recordings

Finally, we compared the characteristics of neurons recorded extracellularly and intracellularly. The difference in distributions of mean phases in L2 and L5 was very similar for both sets of neurons. The majority of neurons recorded extracellularly in both L2 and L5 had their mean phase of activity when their corresponding ventral root was most active (71% of L2 neurons, 64% of L5 neurons). For intracellularly recorded neurons, 92% of L2 neurons were active when L2 was most active, whereas 63% of L5 neurons fired when L5 was active. However, the difference in the R values in the two segments appeared to differ between neurons recorded extracellularly and neurons recorded intracellularly. For extracellularly recorded neurons, L2 neurons had a mean R value that was 0.13 larger than that of L5 neurons. For intracellularly recorded neurons, L3 neurons had a mean R value that was only 0.02 larger than that of L5 neurons. Thus it appeared that the difference between L2 and L5 modulation strength was larger for neurons recorded extracellularly than for those recorded intracellularly.

Discussion

In the present study we used a combination of extracellular and intracellular techniques to quantify the locomotor related spike activity of neurons located in the ventromedial regions of a rostral (L2) and caudal (L5) lumbar spinal segment of the neonatal rat. The ventromedial regions of the neonatal rat spinal cord are necessary for the production of rhythmic motor outputs (Kjaerulff and Kiehn 1996), and neurons in these regions show properties appropriate for their involvement in the production of locomotion (Hochman et al. 1994; Kiehn et al. 1996). The activity of neurons in this region of the spinal cord is therefore likely to play an important role in the production of locomotion in the neonatal rat.

However, it is likely that neurons in this region are not a homogeneous population, consisting of neurons with direct projections to motoneurons, ascending, and/or contralateral projections (Eide et al. 1999; Jankowska 1992). Because we did not identify the projection patterns of individual neurons, we expect that the results described here reflect the properties of a heterogeneous population of neurons. It would therefore be interesting in future experiments to compare the firing properties of different classes of neuronal populations.

Despite this potential heterogeneity, this study showed that rostral and caudal neural populations displayed several distinct characteristics. First, the majority of L2 neurons was active when the L2 ventral root was active, whereas the majority of L5 neurons was active when the L5 ventral root was active. L5 neurons, however, had a wider distribution of their preferred phases of activity throughout the locomotor cycle than L2 neurons. Second, we found that the modulation strength of L2 neuronal activity was larger than that of L5.

Methodological considerations

The use of tetrodes in the spinal cord is new. We therefore compared the results obtained using this method with the results obtained using intracellular tight-seal whole cell recordings, a method previously used to examine the activity of spinal interneurons in this preparation (Hochman et al. 1994; Kiehn et al. 1996; Raastad et al. 1996). The two methods would be expected to have different sampling biases: the low-impedance tetrode recordings are likely to sample primarily large neurons, whereas the patch electrodes, with the impedances used here, tend to sample small neurons (Kiehn et al. 1996). It appeared that the neurons sampled with these two methods were similar in terms of where in the locomotor cycle they were active, but there appeared to be differences between their modulation depths. Given the biases of the two methods described above, our observations suggest that the difference between the modulation depth in L2 and L5 might primarily be for larger neurons. Future experiments will be required to examine this possibility in more detail.

Relationships between neuronal activity and the rhythrogenic capability of spinal segments

Several studies have suggested that, although every lumbar segment may be capable of producing some rhythmic motor output in the neonatal rat, rostral segments produce a more stable and a better quality rhythm than caudal segments (Cowley and Schmidt 1997; Kjaerulff and Kiehn 1996; Kremer and Lev-Tov 1997). These observations suggest that the neuronal networks controlling hindlimb locomotion in the neonatal rat are distributed along the length of the lumbar spinal cord (and possibly into the lower thoracic cord) (Cowley and Schmidt 1997) but with a rostrocaudal gradient of their rhythrogenic capability. Experiments in other vertebrates have also suggested such a rostrocaudal distribution in the rhythrogenic capability of the spinal cord (Deliagina et al. 1983; Ho and O’Donovan 1993; Mortin and Stein 1989; see Kiehn and Kjaerulff 1998). The observation in the present study that the depth of modulation of the L5 ventral root was lower than that of the L2 ventral root was consistent with these previous results.

The results of the present study extend these behavioral results by describing a neurophysiological correlate of this rostrocaudal distribution of rhythrogenic capability. In particular, the stronger rhythrogenic capability of rostral segments as compared with caudal segments was paralleled by a stronger rhythmic signal in L2 interneurons as compared with L5 interneurons. This stronger rhythmic signal was due both to a stronger modulation of individual L2 interneurons and to a more concentrated distribution of L2 interneuronal activity within the locomotor cycle. Although it is impossible to directly explain behavioral aspects based on aspects of individual neurons without a detailed knowledge of spinal locomotor networks, the results described here provide a clear parallel between the rostrocaudal distribution of rhythrogenic capability and the rostrocaudal distribution of interneuron firing patterns.

Colocalization of interneuronal and motoneuronal systems

Our results also have implications as to the organization of locomotor networks in the spinal cord. A priori, one would expect that the activity of the neuronal population responsible for a particular behavioral component should reflect the particular characteristics of that component. In the present study we found that the lower depth of modulation of the L5 ventral root was best reflected in the activity of the L4 neuronal population. Conversely, the stronger rhythmic activity of the L2 ventral root was best reflected in the activity of the L2
neuronal population. These results suggest that the locomotor-related ventral root activity of a spinal segment is primarily subserved by interneuronal systems located within the same segment. Because motoneurons projecting in the ventral root of a spinal segment in the neonatal rat are predominantly located within the same segment (Eide et al. 1999), this conclusion implies a colocalization of motoneurons and the neuronal networks responsible for their activation during locomotion. Such a hypothesis, that the interneuronal systems responsible for the production of rhythmogenic motor output are located near the motoneurons that they control, has been proposed in other systems as well (Cheng et al. 1998; Mortin and Stein 1989). Also consistent with this idea, the majority of interneurons projecting monosynaptically to motoneurons in the adult rat are innervated (Puskar and Antal 1997).

The conclusions described above do not imply that there is no interaction between the locomotor networks located in different segments of the spinal cord. Clearly, to produce a stable alternating rhythm, the neural networks responsible for different segments of the spinal cord. Because motoneurons projecting in the ventral root of a spinal segment in the neonatal rat are predominantly located within the same segment as the motoneurons that they innervate (Puskar and Antal 1997).

The differences in L2 and L5 interneuron firing might therefore be unrelated to the rostrocaudal gradient of rhythmogenic capability in the spinal cord. Given the larger number of motor pools in the L5 spinal segment (Nicolopolous-Stournaras and Iles 1983), one might expect the motor output of L5 to have a wider distribution of activity than that of L2 and interneurons in L5 would consequently be expected to have a wider range of activity than interneurons in L2. Thus, colocalization might be the explanation for the differences in the distributions of mean phases of neurons in L2 and in L5. This possibility is difficult to evaluate definitively because the precise distribution of motor pools in the spinal cord and their activation patterns during locomotion are not completely determined for this preparation (see, however, Kiehn and Kjaerulff 1996; Nicolopolous-Stournaras and Iles 1983). It nonetheless seems possible that the difference in the distributions of preferred phases in L2 and L5 interneurons reflects a difference in the distributions of motor pools in these different segments.

However, the difference in the modulation depths of interneurons in rostral and caudal spinal segments is more difficult to explain based on particular characteristics of motor pools in rostral and caudal spinal segments. We excluded the possibility that the differences were due to differences in the properties of flexor- or extensor-related activity: L5 neurons active in flexion had similar R values to L4 neurons active in extension. Another possibility is that this difference might result from L5 containing more motoneurons innervating bifunctional muscles. Because these muscles in some cases fire with multiple peaks (Kiehn and Kjaerulff 1996), we might expect neurons involved with these muscles to be active over a wider range of the cycle and therefore have lower R values. However, the bifunctional muscles that are known to be contained in L5 (biceps femoris and semitendinosus) (Nicolopolous-Stournaras and Iles 1983) are mainly active as pure extensors with the methods used in this study (Kiehn and Kjaerulff 1996). L5 also contains a significant proportion of bifunctional motoneurons, such as those innervating the different heads of quadriceps (Nicolopolous-Stournaras and Iles 1983). Further, the differences in the depth of modulation of neurons cannot simply be explained by a longer period of activity through the locomotor cycle, because the peak to valley of neural activity should be largely independent of the width of firing. More generally, the observation that the modulation strength of neurons in L3 and in L6 also parallels the rostrocaudal gradient described for L2 and L5 suggests that firing in rostrally and caudally located interneurons is not due simply to differences in the particular motoneuron populations in the different spinal segments.

Gradient of rhythmogenic capability or of motoneuron pools?

If such a colocalization of motoneurons and associated interneurons in the spinal cord is in fact the case, it might be that the characteristics of interneuronal activity described here reflect the characteristics of the motor pools with which they are associated rather than an interneuron’s rostrocaudal location. The differences in L2 and L5 interneuron firing might therefore be unrelated to the rostrocaudal gradient of rhythmogenic capability in the spinal cord. Given the larger number of motor pools in the L5 spinal segment (Nicolopolous-Stournaras and Iles 1983), one might expect the motor output of L5 to have a wider distribution of activity than that of L2 and interneurons in L5 would consequently be expected to have a wider range of activity than interneurons in L2. Thus, colocalization might be the explanation for the differences in the distributions of mean phases of neurons in L2 and in L5. This possibility is difficult to evaluate definitively because the precise distribution of motor pools in the spinal cord and their activation patterns during locomotion are not completely determined for this preparation (see, however, Kiehn and Kjaerulff 1996; Nicolopolous-Stournaras and Iles 1983). It nonetheless seems possible that the difference in the distributions of preferred phases in L2 and L5 interneurons reflects a difference in the distributions of motor pools in these different segments.

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Mechanisms responsible for the rostrocaudal gradient of rhythmic modulation

The above considerations suggest that the properties of interneuron firing described here are difficult to explain based simply on characteristics of the motoneuron pools in different segments of the spinal cord. In particular, the modulation depth of spinal interneurons appears to be best related to rostrocaudal location rather than to the features of the motoneuron pools in different regions of the spinal cord. However, we emphasize that this study only describes a correlation between the activity patterns of spinal interneurons and the rostrocaudal gradient of rhythmogenic capability in the spinal cord. This study has not attempted to identify the particular mechanism responsible for this gradient or its reflection in the activity patterns of spinal interneurons. There are potentially many different mechanisms that might be responsible for this rostrocaudal gradient, and we discuss only a few of them below.

One possible explanation for the differences in the characteristics of rostral and caudal spinal interneurons is that the intrinsic membrane properties of individual rostral neurons might confer to them a large modulation strength. For example, neurons with strong oscillatory properties generally have a large modulation strength of their spike activity (Kiehn et al. 1996). It would therefore be interesting to examine whether there was a difference between rostral and caudal neurons in the oscillatory properties of neurons in the ventromedial regions (Hochman et al. 1994; Kiehn et al. 1996). Another possibility is that the proportion of neurons directly involved in producing locomotion is higher in L3 than in L5, resulting in a clearer rhythmic signal in the L3 neural activity. Finally, the weaker modulation of L4 neurons might reflect properties of the interactions within the network of interconnected L5 interneurons. For instance, inappropriate or weak inhibitory cou-
pling between interneurons might result in a lower modulation of the synaptic drive to individual interneurons. Such a difference in the inhibitory systems of rostral and caudal segments of the spinal cord has in fact been described in the tadpole spinal cord (Yoshida et al. 1998). Future experiments will be required to establish the precise mechanism or mechanisms responsible for the rostrocaudal gradient of rhythmogenic capability in the spinal cord.

Conclusions

In this study we quantified the locomotor-related spike activity in interneurons located in rostral and caudal segments of the neonatal rat lumbar spinal cord. We found that the rhythmic signal of rostral neuronal populations was stronger than that of caudal populations, paralleling the differences between the rhythmogenic capabilities of rostral and caudal segments described previously. These results further suggested that the interneuronal systems related to the activity of a ventral root during locomotion are localized in close proximity to the motoneurons projecting in that ventral root. This study therefore provides evidence consistent with previous studies suggesting that the networks producing hindlimb locomotion in the neonatal rat spinal cord are not restricted to a few segments (Cazalets et al. 1995) but are distributed throughout the extent of the spinal cord.

These experiments also describe the first use of tetrodes to record from the activity of spinal neurons of which we are aware. This method can potentially increase the numbers of neurons examined in a particular study or examined simultaneously. The examination of neuronal systems at a population level has advanced the understanding of systems such as simple withdrawal reflexes (Lewis and Kristan 1998), spatial navigation (Wilson and McNaughton 1993), and the production of voluntary movements (Georgopoulos et al. 1988). It seems likely that similar analyses will help elucidate the operation of the neuronal networks producing locomotion as well.

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