Flexibility of Motor Pattern Generation Across Stimulation Conditions by the Neonatal Rat Spinal Cord

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INTRODUCTION

Circuitry in the spinal cord is capable of producing complex rhythmic motor outputs closely resembling locomotion produced in the intact nervous system (Grillner 1981). These spinal central pattern generators are present at birth (Cazalets et al. 1992; Kiehn 2006; Kudo and Yamada 1987; Smith and Feldman 1987) and, although not fully mature until a few weeks postnatal (Westerga and Gramsbergen 1990), produce muscle activation patterns similar to locomotor patterns produced by adults (Cowley and Schmidt 1994; Hayes et al. 2009; Juvin et al. 2007; Kiehn and Kjaerulff 1996). Using such neonatal preparations isolated in vitro, recent studies have made considerable progress in our understanding of pattern generation by spinal motor systems (Brownstone and Wilson 2008; Kiehn 2006; Whelan 2003). In such experiments, the motor pattern is often characterized by recording selected ventral roots and evoked patterns are considered to be “locomotor-like” if there is an alternation between the mainly flexor related activity on ipsilateral L₂ and the mainly extensor activity on L₅ ventral roots and between contralateral ventral roots at the same spinal segment. Such ventral root characterizations are useful since they provide a simple way to monitor rhythmic activity produced by spinal circuitry.

However, even in the neonate, spinal circuitry recruits muscles in a more complicated pattern during the evoked rhythm than simple alternation of flexors and extensors with many muscles showing activity only in a portion of flexion or extension or showing activity across the transitions between flexion and extension (Kiehn and Kjaerulff 1996). Further, activation of spinal circuitry by different pharmacological agents evokes distinct patterns of muscle activations. In particular, when comparing the patterns evoked by serotonin (5-HT) and dopamine (DA), the activation of several muscles was altered so that muscle activation patterns after DA application showed double bursting or switched their activation between flexion and extension more frequently (Kiehn and Kjaerulff 1996). This work suggests that not only is spinal circuitry capable of producing complex motor patterns but that this patterning is flexible, with muscles able to shift their roles depending on the neuromodulatory state of the system.

In addition to pharmacological activation, there are several other ways to evoke locomotor-like rhythmic patterns in this preparation. In particular, electrical stimulation of inputs to the spinal cord, either by stimulation of dorsal (Marchetti et al. 2001) or ventral (Mentis et al. 2005) roots, of the cauda equina (CE) (Lev-Tov et al. 2000; Strauss and Lev-Tov 2003), or of descending brain stem (Zaporozhets et al. 2004) pathways is commonly used to evoke rhythmic patterns. This stimulation is potentially more physiologically interpretable as compared with the bath application of pharmacological agents, relying on the synaptic connectivity of those inputs to spinal interneurons rather than the global activation of neurons by bath-applied agents.

Although both pharmacological and electrical stimulation are commonly used to characterize motor rhythms in the neonatal rodent, a systematic comparison between their evoked muscle activation patterns has not been performed. Such a comparison is obviously important for the interpretation of the results of experiments using the different techniques: if stimulation paradigms access spinal circuitry differentially, then the results obtained using one might be difficult to generalize
to the other. In addition, the differential activation of spinal circuitry might be useful to characterize the role of interneuronal populations in rhythm generation. In the present study, we use the in vitro spinal cord with hindlimb-attached preparation to examine the muscle activation patterns evoked by different stimulation paradigms. This preparation allows us to isolate spinal circuitry from the rest of the brain and to record the activity in several muscles simultaneously during rhythmic motor activity. We find that there are in fact systematic differences in the patterns evoked by these different stimulus conditions, both in terms of the phasing of individual muscles and in terms of their overall statistics.

Methods

Experimental preparation

Twenty-five neonatal rats (P0–P5) were used in these experiments. All procedures were approved by the Animal Care and Use Committee at Northwestern University. Neonates were anesthetized with isoflurane until unresponsive, decapitated, and eviscerated under ice-cold oxygenated low-calcium Ringer solution [containing (in mM) 118–28 NaCl, 4.7 KCl, 25 NaHCO3, 1.2 KH2PO4, 3.5 MgSO4, 0.25 CaCl2, and 20 glucose; pH 7.4, 300–330 osM]. The spinal cord was exposed by ventral laminectomy, and the left side of the body was removed, cutting along the leftmost edge of the spinal column and through the torso. The dura mater was then opened with iridectomy scissors, and all dorsal and ventral roots rostral to L1 were severed. Starting at the rostral end, the spinal cord was gently freed of the vertebral column by tearing and cutting away connective tissue. The vertebral column was then severed near the caudal edge of the rib cage, and the rostral body was removed, leaving the entire spinal cord with innervated right hindlimb intact. To increase the perfusion to the spinal cord, the dorsal portion of the vertebral column was also removed so that in the end ~2/3 of the vertebral column surrounding the lumbar and sacral spinal cord was removed. The preparation was then transferred to a chamber continuously perfused with the same cold, low-calcium Ringer solution as used in the initial dissection. The skin overlying the hindlimb and underlying connective tissue were then removed. In all preparations, both heads of the gracilis muscle were removed to give access to the underlying muscle groups. The preparation was then immobilized using insect pins through the vertebrae, pelvis, femur, and tibia/fibula, and contralateral dorsal and ventral roots were pinned out to increase the space between the spinal cord and vertebral column for better perfusion. Any remaining dorsal or ventral roots not associated with the lumbar spinal cord were cut at this time. The perfusing medium was then switched to 100%-calcium Ringer solution (same concentrations as in the preceding text, but with MgSO4 and CaCl2 concentrations adjusted to 1.25 and 2.5 mM respectively). Suction electrodes were then placed on selected muscles of the hindlimb. In this study, we report results obtained from recordings of iliopsoas (IP), rectus femoris (RF), semimembranosus (SM), adductor magnus (AM), and semitendinosus (ST). IP is a pure hip flexor, RF is a knee extensor and hip flexor, SM is a hip extensor and knee flexor, AM is a hip extensor, knee flexor, and limb adductor, and ST is a hip extensor and knee flexor (Greene 1935). Nerve cuts to individual muscles confirmed that the cross-talk between recordings of the muscle activity in this preparation was negligible and had little impact on the results of these experiments. We also regularly observed periods of independent activity in muscles, further suggesting a minimal effect of cross-talk between muscle recordings. In some animals, the hindlimb was also deafferented by cutting the lumbar dorsal roots. Muscle activity was preamplified (×10 gain, A/M systems model 3600 headstage) near the preparation, filtered (10–1,000 Hz) and acquired at 2,500 Hz for subsequent data analysis using custom-written software in LabView.

Rhythmic motor activity was evoked in three different ways. A combination of serotonin (5-HT; 4–10 μM) and N-methyl-D-aspartic acid (NMDA; 4–10 μM) was used to evoke rhythmic activity. We also used stimulation of sacrocaudal afferents by placing the CE of the spinal cord up to the S4 roots and including the conus medullaris in a suction electrode and applying electrical stimulation (6 Hz for 20–25 ms; pulse duration: 250 μs, 10–200 μA). We used similar parameters to electrically stimulate the contralateral L5 dorsal root to evoke motor rhythms. The stimulation intensity (concentrations of 5-HT/NMDA, level of current for CE and L5 stimulation) were adjusted as necessary throughout the experiment to evoke stable, consistent rhythmic patterns of activity in the dissected hindlimb. Stimulation artifacts were removed in off-line analysis.

Data processing and analysis

Onsets of IP muscle activity were identified manually using custom software written in Matlab. IP onsets were used to define the cycle as they were the most consistent and were generally reliably estimated (see examples in Figs. 1, 6, and 8). To ensure that analyzed patterns were locomotor-like as might be assessed by ventral root recordings, we identified cycles in which distinct bursts (~100-ms duration) of both IP and SM and/or AM were present as assessed by visual inspection. Although in many cases of both drug and electrical stimulation cycles meeting these criteria were produced in continuous periods (see Figs. 1, 6, and 8), we included cycles in subsequent analyses whether or not they were part of such a continuous sequence. The identified cycles were then extracted across all channels, each individual cycle was divided into 100 bins, and the activity of each muscle was integrated in each bin.

To determine whether a muscle was modulated during an individual cycle, we used a modified version of standard circular statistics (Berens 2009; Berkowitz and Stein 1994a, b; Drew and Doucet 1991; Mardia 1972; Tresch and Kiehn 1999). The mean resultant length of muscle activation was calculated using the activity of the muscle during each bin of the cycle. If a muscle had integrated activity ai for the ith bin of the cycle corresponding to phase qi, then the mean resultant length is calculated as

\[
R = \frac{1}{\sum a_i} \left( \sum a_i \cos q_i \right)^2 + \left( \sum a_i \sin q_i \right)^2
\]

The mean resultant length (R) of a muscle reflects the dispersion of activity across the cycle: a value near 1 implies that the activity is highly concentrated in the cycle, whereas a value near 0 implies that the activity is uniform or symmetrically distributed throughout the cycle. To evaluate whether the activity of a muscle for a particular cycle was well modulated, we performed a bootstrap statistical test. The activity of the muscle on the cycle was randomly shuffled across bins and the resulting mean resultant length was calculated. This process was repeated 1,000 times to create a distribution of the mean resultant lengths that would be expected if there were no relation between the activity of the muscle and the rhythm. If the value of the mean resultant length that was actually observed was large with respect to this distribution (>95% of the distribution), we concluded that the muscle was tuned during that particular cycle of the evoked rhythm. Only cases in which at least six tuned cycles for a particular muscle were observed were included in subsequent analyses of that muscle. The preferred phase θ of a muscle on a particular cycle was calculated as

\[
\theta = \tan^{-1} \frac{\sum a_i \sin q_i}{\sum a_i \cos q_i}
\]

with terms as defined in the preceding text. We also calculated the modulation depth of individual muscles on each cycle. The modulation depth was calculated as mod = (peak – valley)/valley, where peak is the activity of the muscle within 30° of phase around the mean
phase of the muscle and valley is the activity of the muscle within 30° around the phase 180° away from the mean phase of the locomotor cycle. Watson-Williams tests were used to evaluate whether distributions of phases were different from one another (Mardia 1972) using the Circular Statistics Toolbox for Matlab (Berens 2009). For measures ranging between 0 and 1 (fraction of tuned cycles, mean resultant lengths), we used a Kruskal Wallis nonparametric test to compare samples. Significance levels were set at $P < 0.05$.

RESULTS

Comparison of 5-HT/NMDA- and CE-evoked rhythms

The combination of 5-HT and NMDA evoked a steady rhythm, consisting of a basic alternation between the hip flexor IP and predominantly hip extensors SM and AM. Although AM and SM have significant actions on other degrees of freedom, we refer to them here as hip extensors for simplicity. These hip flexor and extensors were consistently activated in alternation with no overlapping activity. In contrast, ST and RF were activated closer to the transitions between bursts. ST started late in extension and usually terminated prior to the onset of flexion. RF started late in flexion and usually terminated prior to the onset of extension. Thus the activation of RF and ST essentially exchanged phases between 5-HT/NMDA and CE patterns. In two animals, we confirmed that the contralateral L3 ventral root was consistently activated out of phase with the ipsilateral IP in all cases of CE stimulation, showing that these patterns qualified as locomotor-like.

The patterns evoked by 5-HT/NMDA and by CE stimulation in one animal are summarized in Fig. 2 (same animal as for the examples shown in Fig. 1). The activity on each muscle was aligned to the onset of IP and expressed relative to a standardized phase, starting at 0° (the onset of the initial IP burst) and ending at 360° (the onset of the subsequent IP burst). The activity of each muscle was then averaged across all cycles and the mean phase of activation for each individual cycle was calculated. Figure 2 shows the average trace for each muscle in the two stimulation conditions with the distribution of mean

![Figure 1](http://jn.physiology.org/). Examples of the rhythms evoked by serotonin/N-methyl-D-aspartate (5-HT/NMDA) application (A) and stimulation of the cauda equina (B). The concentration of 5-HT/NMDA was 7.5/6 M in A. The stimulation strength in (B) was 165 μA. Data are taken from the same animal and the display gains are the same for both conditions.
phases across individual cycles shown underneath each trace. This figure clearly shows the difference in the activation of RF and ST across the stimulus conditions. Closer examination of Fig. 2 also suggests a difference in the consistency of the patterns evoked in the two conditions. In particular, the distributions of mean phases for SM, AM, and RF in this animal appear to be more broadly distributed in the CE-evoked patterns than in the 5-HT/NMDA-evoked patterns. This observation suggests that CE-evoked patterns were less consistent across cycles than 5-HT/NMDA-evoked patterns. We examined these differences in more detail in the analyses described in the following text.

We first examined the differences in the phasing of muscles in the two stimulation conditions. Figure 3, A and B, shows the distribution of preferred phases for muscles in each stimulation condition. Each count in these distributions represents the mean phase observed for one animal (the average of the mean phases of individual cycles illustrated in Fig. 2). Consistent with the examples shown in Figs. 1 and 2, the distributions of IP, AM, and SM were not significantly different (Watson-Williams test, $P > 0.05$) between 5-HT/NMDA and CE. In contrast, both RF and ST were significantly different between the two stimulus conditions: the mean phase of RF was 114.11° in 5-HT/NMDA and was 287.14° in CE, whereas the mean phase of ST was 302.04° in 5-HT/NMDA and was 74.00° in CE ($P < 0.05$). These differences were also regularly observed within individual animals when both stimulus conditions were tested (Fig. 3C); 14/15 cases of RF and 10/12 cases of ST showed a significant alteration, whereas only 5/18 cases of IP, 8/17 cases of SM, and 5/17 cases of AM showed a significant change in phasing across conditions (the difference in total number of cases examined reflects the fact that in individual animals not every muscle produced enough tuned cycles to allow this comparison to be made; see METHODS). Thus the activation of RF and ST were selectively and consistently altered between these two stimulus conditions.

The activity of individual muscles was more reliably modulated during 5-HT/NMDA patterns. Figure 4 shows the distribution for all animals of the fraction of cycles for which each muscle was significantly modulated. The fraction of unimodally tuned cycles was significantly higher for IP, SM, RF, and ST in 5-HT/NMDA rhythms than in CE rhythms (0.99 vs. 0.96 for IP, 0.99 vs. 0.89 for SM, 0.94 vs. 0.66 for RF, and 0.97 vs. 0.63 for ST). AM was not significantly different (0.94 vs. 0.89). Note also that the difference in the fraction of tuned cycles was especially large for both RF and ST.

Considering only those cycles that were significantly modulated during the cycle (and which therefore had a well-defined preferred phase for that cycle), the phase of activation of muscles was considerably less consistent in CE than in 5-HT/NMDA activity. We quantified this consistency by calculating the mean resultant length of the distribution of phases for each animal, then examined whether across animals this measure was close to 1 (indicating a more consistent phasing of mus-
cles) for 5-HT/NMDA versus CE. This measure quantifies the dispersion of muscle phasing from cycle to cycle for a given animal. Figure 5 shows the dispersions for each muscle across the two conditions. The average mean resultant length in 5-HT/NMDA was 0.95 for IP, 0.91 for AM, 0.92 for SM, 0.89 for RF, and 0.92 for ST. The mean resultant length in CE was 0.91 for IP, 0.79 for AM, 0.83 for SM, 0.71 for RF, and 0.55 for ST. In each case the dispersion was greater (lower mean resultant length) in CE evoked patterns ($P < 0.05$), indicating that the phasing of muscles was significantly less consistent.

We also examined the modulation depth of the evoked patterns. The modulation depth was calculated as the difference between the activity of the muscle near its peak and its activity near its valley for each cycle. There was not a significant difference in the modulation depth across the two stimulus conditions for most of the muscles considered (for IP: 5.57 vs. 7.62, SM: 4.00 vs. 4.80, RF: 2.41 vs. 3.10, ST: 1.89 vs. 1.85 in 5-HT/NMDA vs. CE; data not shown). Only AM showed a significantly higher modulation in CE-evoked patterns (2.88 vs. 4.21).

Finally, there was no significant difference between the cycle duration of 5-HT/NMDA and CE rhythms. The mean duration of 5-HT/NMDA rhythms was 2.55 ± 0.90s and for CE rhythms was 2.15 ± 0.36s ($P > 0.05$).

Difference in patterns is preserved after deafferentation

In the experiments described in the preceding text, the hindlimb afferents from the limb were left intact and the limb was re-stretched. The differences between 5-HT/NMDA and CE described in the preceding text could therefore have reflected a difference in how the two stimulus conditions utilized sensory information from the hindlimb. To evaluate this possibility, we repeated the experiments in animals following deafferentation at the lumbar level. Because all other dorsal roots were already...
severed in these preparations, this lumbar deafferentation eliminated all sensory information reaching the spinal cord through dorsal roots.

An example of the patterns evoked in 5-HT/NMDA and CE stimulation is shown in Fig. 6, demonstrating that the characteristic phasings of muscles in each pattern were preserved following deafferentation. Figure 7, A and B, illustrates the distributions of preferred phases for each muscle in the different stimulus conditions. As suggested by the figure, the phasing of RF and ST was significantly different between stimulus conditions. The mean phase of RF was 110.67° in 5-HT/NMDA and 269.38° in CE stimulation, whereas the mean phase of ST was 297.46° in 5-HT/NMDA and 91.19° in CE. Similarly, the phasing of IP, AM, and SM were not significantly changed between 5-HT/NMDA and CE. This consistency was also observed when the effects of deafferentation were examined in

**FIG. 6.** Example of the patterns evoked following deafferentation for 5-HT/NMDA (A) and CE stimulation (B). Concentrations of 5-HT/NMDA in A were 77 μM. Stimulation strength in B was 80 μA.

**FIG. 7.** Summary of the phasing of each muscle in 5-HT/NMDA (A) and CE (B) following deafferentation. Conventions the same as Fig. 3.
individual animals (Fig. 7C). In 8/8 cases for RF and 7/7 cases for ST, there was a significant difference in the phase of the muscle between the two stimulus conditions. In contrast, only 3/8 cases for IP, 3/8 cases for AM, and 4/8 cases for SM showed a significant change in phasing.

Similarly, the generally more variable tuning of muscles in CE as compared with 5-HT/NMDA was also observed following deafferentation. As observed with afferents intact, RF and ST each had fewer tuned activations during CE activity than during 5-HT/NMDA activity (for RF 0.89 vs. 0.70, for ST 0.94 vs. 0.65, in 5-HT/NMDA vs. CE; not shown). IP, AM, and SM were not significantly different. Similarly, the mean resultant length of each muscle was still significantly larger in 5-HT/NMDA for all muscles (for IP 0.98 vs. 0.89; AM 0.93 vs. 0.78, SM 0.95 vs. 0.81, RF 0.93 vs. 0.77, ST 0.92 vs. 0.60 in 5-HT/NMDA vs. CE; not shown), indicating a wider dispersion of phasing of each muscle in CE-evoked patterns. Finally, no muscles showed significantly different modulation depth when comparing 5-HT/NMDA and CE patterns (data not shown).

Taken together, these results demonstrate that the differences in patterns observed across stimulus conditions were not a consequence of a differential sensitivity to afferent processing in the two conditions. Instead these differences reflect basic differences in the muscle activation patterns generated by spinal circuitry by the two stimulation paradigms.

**Comparison to rhythmic activity evoked by dorsal root stimulation**

The preceding results showed consistent differences in the phasings and consistency of the rhythms evoked by 5-HT/NMDA and CE stimulation. We next examined whether similar differences could be observed when considering patterns evoked from electrical stimulation of a different pathway. In particular, we examined the rhythmic muscle activation patterns evoked by contralateral dorsal root stimulation.

Figure 8 shows an example of the rhythmic activity evoked by stimulation of the contralateral L5 dorsal root. As was the case for the 5-HT/NMDA and CE-evoked patterns, the L5-evoked patterns consisted of a basic alteration between IP and AM/SM. However, in these patterns, ST was active mainly during extension while RF was only weakly activated during flexion. Comparison of this example to those shown in Fig. 1 suggests that the phasing of muscles evoked by L5 stimulation was more similar to those evoked by 5-HT/NMDA than to those evoked by CE stimulation. Indeed, none of the muscle phasings (Fig. 9A) were significantly different between 5-HT/NMDA and L5. When comparing L5 and CE stimulation responses, although there was no significant difference among the phases of IP, AM, or SM, the phases of RF and ST were both significantly different (RF: 287.14° vs. 69.42°, ST 73.43° vs. 302.04° for CE vs. L5). Similar to the patterns evoked by 5-HT/NMDA and CE, the patterns evoked by L5 stimulation were preserved following deafferentation (data not shown).

Although the preceding results suggest that L5 stimulation evokes a pattern similar to that evoked by 5-HT/NMDA, there were also substantial differences between the two patterns when considering their reliability. Like CE-evoked patterns, L5 patterns were considerably less reliable than 5-HT/NMDA patterns. The fraction of tuned cycles was consistently lower ($P < 0.05$) in L5-evoked patterns for IP, SM, RF, and ST (IP 0.96; SM 0.95, RF 0.20, ST 0.89) when compared with 5-HT/NMDA patterns. AM was not significantly different between the two patterns. Similarly, the phasing of all muscles were more dispersed ($P < 0.05$) in L5 patterns (IP 0.92; AM 0.84, SM 0.88, RF 0.64, ST 0.82) when compared with 5-HT/NMDA patterns. When comparing L5 and CE patterns, the reliability of IP, SM, and AM was not significantly different as assessed by either the fraction of modulated cycles or the dispersion of preferred phases. However, there were significant differences in the reliability of RF and ST activations between L5 and CE evoked patterns. As expected from Fig. 8, RF was less likely to be modulated in L5 patterns than in CE patterns ($P < 0.05$). However, the opposite was true for ST as it was more likely to be modulated in L5 patterns than in CE patterns ($P < 0.05$). Similarly, the dispersion of preferred phases was larger for ST in CE patterns than in L5 patterns ($P < 0.05$), although there was no difference in the dispersion of RF preferred phases. Finally, the modulation depth was not different for any muscle between either L5 and 5-HT/NMDA or between L5- and CE-evoked patterns.

**Comparing intra stimulus patterns before and after deafferentation**

As described in the preceding text, the basic differences between patterns were maintained following deafferentation. These comparisons do not exclude potential effects of deafferentation.

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**Figure 8.** Example of the pattern evoked by stimulation of the contralateral L5 dorsal root at a stimulations strength of 42.5 µA.
entation on the evoked patterns but only show that any such changes were not large enough to obscure the systematic differences between stimulation conditions. However, even when comparing the same stimulus condition before and after deafferentation, we did not observe consistent effects for any of the measures considered here. There were no significant differences in the phasing of any muscle before and after deafferentation for any of the stimulus conditions. When considering the fraction of modulated cycles, only AM in CE-evoked patterns showed a significant increase in the fraction of tuned cycles following deafferentation (0.89 vs. 0.97). Similarly, there were no consistent differences in the dispersion of preferred phases for any of the muscles in any stimulus condition. These results suggest that afferent feedback played a minimal role in regulating these characteristics of evoked patterns under the experimental conditions examined here.

**DISCUSSION**

This study demonstrates the systematic differences between the rhythmic muscle activation patterns evoked by different stimulus conditions in the isolated neonatal rat spinal cord. We showed that the phasing of muscles was altered between conditions with the activity of RF shifting from late flexion in 5-HT/NMDA-evoked patterns to late extension in CE-evoked patterns, whereas the activity of ST shifted from late extension in 5-HT/NMDA-evoked patterns and L₅ to flexor related activity in CE-evoked patterns. In L₅-evoked patterns, activation of RF was usually absent or weak. There were also substantial differences in the quality of the rhythms evoked in each condition with a higher likelihood of muscles being tuned to the rhythm in 5-HT/NMDA than in either CE- or L₅-evoked rhythms. Similarly, there was a looser relationship between the activity of several muscles and the cycle in either CE- or L₅-evoked patterns when compared with 5-HT/NMDA-evoked patterns. These differences were largely preserved after deafferentation, suggesting that they represented fundamental intrinsic differences in the activation of spinal neural circuitry underlying the generated patterns. These results suggest that the neonatal rat spinal cord is capable of producing a range of rhythmic motor patterns, whether they are evoked by pharmacological neuromodulation or by electrical stimulation of afferent pathways.

**Alterations in the phasing of muscle activations**

The main result of the present experiments was that activation of spinal cord circuitry by different pathways evokes rhythms with different coordination patterns among hindlimb muscles. It is important to note that we only included cycles in which both flexors (IP) and extensors (SM and/or AM) were active. We used this criterion so that the observed patterns could be considered to be locomotor-like according to standard definitions based on ventral root recordings. The consistent alternation between IP and SM/AM reported here is thus an expected reflection of the inclusion criteria for analysis. However, the dramatic alteration of RF and ST activations, with the two muscles essentially switching places between CE and 5-HT/NMDA rhythms, was not expected. Similar phase shifts have been reported when comparing patterns evoked by 5-HT and by DA in the neonatal rat with quadriceps and hamstring muscles patterns being especially flexible (Kiehn and Kjaerulff 1996). The present study extends that work to demonstrate that such flexibility does not depend on the use of pharmacological agents that might impose a global neuromodulatory state across the entire spinal cord. Instead these results suggest that such flexibility can also be observed when spinal interneuronal systems are activated synthetically by afferent stimulation. Importantly, the patterns evoked by stimulation of CE and L₅ afferents were also distinct from one another, demonstrating that the different patterns were not simply due to pharmacological as opposed to electrical stimulation. And although similar changes in the phase relationships of RF have been demonstrated in the neonatal rat due to alterations in afferent feedback, the changes in phasing observed here were preserved following deafferentation (Hayes et al. 2009). It is important to note that in the Hayes et al. study, afferent feedback included information about a moving limb, as well as tactile sensory
feedback from rhythmic interaction with a treadmill. In our study, afferent feedback is expected to be considerably less influential because the limb is fixed. Nevertheless our results clearly demonstrate that this flexibility is an intrinsic feature of spinal central pattern generators in the neonatal rat.

Although it is clear that stimulation of different pathways evoked distinct motor patterns, the exact behavioral correlate of each of these patterns is less clear. Previous work has suggested that the late flexion activity of RF observed in 5-HT rhythms (and in the 5-HT/NMDA rhythms observed here) is most consistent with swimming motor patterns (de Leon et al. 1994; Guran and Altman 1980), while the more extensor dominated or double-bursting RF activity observed in DA, similar to that reported here for CE stimulation, is more consistent with locomotor patterns (Guran et al. 1980; Kiehn and Kjaerulff 1996; Nicolopoulos-Stournaras and Iles 1984). However, late flexor bursts in RF can also be observed in the locomotor patterns evoked by MLR and hypothalamic stimulation as well (Goudard et al. 1992). Similarly, although the flexor related activity of ST observed in CE patterns could be considered as being consistent with locomotion (Guran et al. 1980; Nicolopoulos-Stournaras and Iles 1984), this activation usually occurred throughout the entire flexion phase rather than only during early flexion as is usually observed during adult locomotion. Because of these and other ambiguities, we have therefore found it difficult to make a clear identification of the patterns evoked here with particular locomotor behaviors. It should be noted that the presence of double bursting was not examined systematically in the present study because the objective identification of distinct bursts was generally difficult because of the very sparse activation of muscles in these patterns. We therefore did not attempt to distinguish between potential behavioral correlates on the basis of double bursting (Kiehn and Kjaerulff 1996). We also note that rhythmic behaviors other than locomotion or swimming can be produced by spinal systems. For example, scratch reflexes in the turtle broadly share many of the features of the patterns observed here, both in that they consist of a basic alternation between hip flexors and extensors and that forms of scratches are differentiated on the basis of phase shifts of other muscles, especially knee flexor and extensor muscles (Robertson et al. 1985). More generally, in the absence of data describing muscle activations during behaviors in the neonate, it is difficult to compare the patterns observed here with natural behaviors.

These results also reinforce the point that characterization of motor rhythms by ventral root recordings can obscure details of motor patterning (Cowley and Schmidt 1994). Although each of the stimulation paradigms examined here evokes locomotor-like patterns when assessed by ventral root recordings, the actual muscle activation patterns were clearly distinct. Studies examining the role of spinal interneurons in pattern generation might be able to exploit this range of motor patterning. For instance, HB9-positive interneurons are mainly active during flexion when activated by 5-HT/NMDA (Brownstone and Wilson 2008; Hinckley et al. 2005; Kwan et al. 2009). If a subpopulation of these neurons shifted its activity to extension in the rhythms evoked by CE (Kwan et al. 2009), it would suggest that this population was related to the activation of RF motor neurons and that HB9 neurons should be thought of as being related to the details of muscle activations during locomotion. On the other hand, if no HB9 neurons shifted their activity, this population might be related to a more abstract “flexor” phase of locomotion, consistent with standard half center hypotheses for locomotor rhythmogenesis (Brownstone and Wilson 2008). Note, however, that the variability in CE evoked patterns means that it will still be useful to monitor muscle activity during the rhythms, to confirm the changes in muscle phasing. The ability to produce a range of different motor patterns has been useful in examining the role of spinal interneurons in turtle scratch reflexes (Berkowitz and Stein 1994a,b; Stein and Daniels-McQueen 2002), and the distinct patterns observed here might similarly be useful in elaborating mammalian spinal interneuronal systems.

**Alterations in the consistency of rhythmic patterns**

In addition to the differences in phasing described in the preceding text, we also found systematic differences in the consistency of the patterns evoked by each stimulus condition. In general, the patterns evoked by electrical stimulation of either sacrocaudal afferents or the L₄ dorsal root were less reliable than those evoked by 5-HT/NMDA activation. This was seen both in terms of how often muscles showed activity that was rhythmically modulated and in terms of the variability of the phase of the muscle activity when it was actually activated. These two measures are clearly related: weaker or less modulated activity will result in a poorly defined phase for a particular muscle. We emphasize that even though the different phasings of muscles indicated in Fig. 3 represented typical CE patterns well, the variability of these patterns often resulted in muscles being activated at widely different points in the cycles, as indicated by the ranges illustrated in Fig. 2. The levels of variability were not altered following deafferentation, suggesting that phasic sensory feedback was not responsible for the decreased consistency in the afferent stimulation evoked patterns.

The reduced consistency of electrical stimulation evoked patterns might be due to a less reliable activation of interneuronal systems. Activation of afferent systems by electrical stimulation will not necessarily resemble the natural activation of afferents but will recruit a mix of sensory signals in a highly synchronized, intermittent pattern. This potentially complicated afferent input might be difficult for spinal interneuronal systems to interpret, resulting in the production of a less consistent pattern. Pharmacological stimulation, on the other hand, might provide a constant level of activation to interneuronal systems and thereby increase consistency. It should be noted, however, that although electrical stimulation evoked less reliable patterns than did 5-HT/NMDA, all of the observed patterns were quite variable especially when compared with adult behaviors. It might therefore be that the variability observed here is a fundamental characteristic of spinal rhythm generating systems in the neonate (Cazalets et al. 1990). Variability is intrinsic to many motor behaviors and rather than reflecting poorly organized motor behaviors, such variability can reflect efficient control strategies (Latash et al. 2007; Valero-Cuevas et al. 2009). Variability is also potentially useful in development, where it can be used to explore the properties of the environment, or perhaps more relevant for the present experiments to explore the properties of the limb and musculature (Pettersson et al. 2003; Tümer and Brainard 2007). The work of Schouenborg and colleagues (2004) manipulating
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