Specificity of intramuscular activation during rhythms produced by spinal patterning systems in the in vitro neonatal rat with hindlimb attached preparation.

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Abstract

In intact adult vertebrates, muscles can be activated with a high degree of specificity, so that even within a single traditionally defined muscle, groups of motor units can be differentially activated. Such differential activation might reflect detailed control by descending systems, potentially resulting from postnatal experience such that activation of motor units is precisely tailored to their mechanical actions. Here we examine the degree to which such specific activation can be seen in the rhythmic patterns produced by isolated spinal motor systems in neonates. We examined motor output produced by the in vitro neonatal rat spinal cord with hindlimb attached. We recorded the activity of different regions within the posterior portion of biceps femoris (BFp; i.e. excluding the anterior/vertebral head). We found that in the rhythms evoked by bath application of 5-HT/NMDA, all regions of BFp were active during extension. However, the regions of BFp were activated in a specific sequence, with the activation of rostral regions consistently preceding those of more caudal regions in both afferented and deafferented preparations. In the rhythms evoked by cauda equina (CE) stimulation, rostral and middle regions of BFp remained active in extension, but the caudal region of BFp was usually active in flexion. Stimulation of L5 and S2 dorsal roots typically evoked rhythms with all regions of BFp active during extension and although the same rostral to caudal sequence of activation observed in 5-HT/NMDA evoked rhythms could also be observed in these rhythms, we also observed cases with reversed sequences, with activity proceeding from caudal to rostral. S2 dorsal root stimulation occasionally evoked rhythms with flexor activity in caudal BFp, similar to CE evoked rhythms. Taken together, these results suggest a high degree of individuated control of muscles by spinal pattern generating networks, even at birth.
Introduction

Central pattern generators within the spinal cord are capable of producing complex rhythmic motor patterns (Grillner 1981; Rossignol 1996). This capability is intrinsic to spinal cord circuitry immediately at birth (Cazalets et al. 1992; Kiehn 2006; Sqalli-Houssaini et al. 1993; Whelan 2003) (Smith and Feldman 1987; Smith et al. 1988) (Kudo and Yamada 1987) and, although the motor patterns are often considered to consist of a simple alternation between flexion and extension, it is clear that muscle activations even in the neonate can be more complex (Grillner 1981; Kiehn and Kjaerulff 1996; Rossignol 1996) (Klein et al. 2010). Muscles can be active across the boundaries between flexion and extension phases or for only a fraction of a phase in the rhythms produced by the isolated neonatal spinal cord (Kiehn and Kjaerulff 1996) (Hayes et al. 2009; Klein et al. 2010). Further, the activity of some muscles can shift depending on the conditions within which the rhythms are produced (Kiehn and Kjaerulff 1996) (Hayes et al. 2009; Klein et al. 2010). For instance, we have shown in the in vitro neonatal rat that ‘bifunctional’ muscles, such as semitendinosus (ST) and rectus femoris (RF) switch between flexor and extensor related activity in the rhythms evoked by application of serotonin (5-HT) and N-methyl-D-aspartic acid (NMDA) as compared to cauda equina stimulation (Klein et al. 2010).

In intact adults, this specificity extends even further, so that in some cases subregions of muscles or individual motor units can be activated differentially (Grillner 1981; Loeb 1990; Windhorst et al. 1989). Specific regions of complex muscles can be activated selectively during different behaviors (Chanaud and Macpherson 1991; English 1984; Hoffer et al. 1987; Loeb 1990; Pratt et al. 1991; Pratt and Loeb 1991; Schieber 1993; Schieber et al. 2001; Widmer et al. 2003; Windhorst et al. 1989) and this differential activation can often be related to the differential biomechanical actions of each region (Carrasco and English 1999; Carrasco et al. 1999; Chanaud et al. 1991a; b). Similar specificity of motor unit activation has been demonstrated in human motor control (Herrmann and Flanders 1998; Reilly and Schieber 2003; Wakeling 2009). This differential activation implies that the neural systems controlling locomotion and other behaviors can be highly precise in intact animals, having access to individual degrees of freedom and being capable of controlling them according to their specific mechanical action.

However, the extent to which such specificity is intrinsic to spinal pattern generators or is present at birth is unclear. For instance, this specificity might reflect the precise actions of descending systems acting to refine relatively course actions of spinal motor systems, with this refinement potentially due to postnatal experience dependent plasticity (Levinsson et al. 1999; Petersson et al. 2003; Schouenborg 2004). Alternatively, this precision might be intrinsic to spinal motor systems immediately at birth,
reflecting a high degree of sophistication of spinal systems and their prenatal development. We consider these issues in the present study by examining the specificity of activation within different regions of a single muscle, biceps femoris, during rhythms produced by the in vitro neonatal rat spinal cord. Biceps femoris in the rat is a complex muscle, with an anterior head originating from spinal vertebrae and inserting on the femur, and a head originating from the pelvis on the ischium and inserting along the length of the tibia (Greene 1935). This pelvic head can be further divided into a ‘posterior’ head with an insertion on the tibia near the knee and an ‘accessory’ head with an insertion distributed along the remaining length of the tibia, although the distinction between these two heads is not always apparent. Experiments performed using the in vitro neonatal rat spinal cord have reported that all regions of biceps femoris are activated during extension within the rhythmic patterns evoked by 5-HT (Kiehn and Kjaerulff 1996), suggesting that neonatal central pattern generators do not show the specificity of muscle activation exhibited in intact adults. The degree of specificity intrinsic to spinal central pattern generators, especially at birth, and whether it extends to the activation of subregions of complex muscles therefore remains unclear.

Methods

25 animals were used in these experiments. All procedures were approved by the Animal Care and Usage Committee at Northwestern University. The spinal cord with attached hindlimb of neonatal rats (P0-P5) was prepared for in vitro experiments, using methods described previously (Klein et al. 2010). Briefly, animals were anesthetized with isoflurane until unresponsive, decaptitated and eviscerated. Under ice cold low calcium Ringer’s solution (118 -128 mM NaCl, 4.7 mM KCl, 25 mM NaHCO3, 1.2 mM KH2PO2, 3.5 mM MgSO4, 0.25 mM CaCl2, and 20 mM glucose; pH 7.4, Osm 300-330), the spinal cord was exposed by ventral laminectomy and all spinal roots except those innervating the right hindlimb were severed. The body except for the right hindlimb was removed. The preparation with the medial side of the hindlimb facing upwards was then transferred to a chamber continuously perfused with low calcium Ringer’s solution. The medial surface of the biceps femoris was exposed by dissecting away the medial musculature (gracilis, adductor magnus, semimembranosus, semitendinosus, caudofemoralis, and gastrocnemius). The perfusing medium was then switched to 100%-calcium Ringer’s solution (same concentrations as above, but with MgSO4 and CaCl2 concentrations adjusted to 1.25 and 2.5 mM respectively). Note that in the rat biceps femoris consists of two main divisions: biceps femoris anterior which originates on spinal vertebra and inserts on the femur near the knee and the remaining part of
biceps femoris (BFp) which originates from the pelvis at the ischium and inserts along the extent of the
tibia (Greene 1935). All recordings reported here were taken from this latter head arising from the
pelvis (BFp). Similar to what has been described in the cat, BFp can be roughly divided into distinct
compartments as indicated by branching patterns of the innervating nerve (Carrasco and English 1999;
Chanaud and Macpherson 1991; Chanaud et al. 1991a) (Figure 1A). In general, three main branches of
the nerve could be consistently observed across animals, although there could be individual differences
in the distinct patterns of branching. We recorded from three regions of BFp, defined roughly according
to the branching patterns of the innervating nerves along the rostral-caudal extent of the muscle: a
rostral region (rBFp), a middle region (mBFp), and a caudal region (cBFp). The activity of each of these
regions was recorded by placing suction electrodes on each region of the muscle. In a few experiments,
we dissected each region from one another to separate them physically and recorded the activity in
each compartment distal to the cut in order to minimize concerns of electrical cross talk. The results of
these experiments (see Figure 1B) were very similar to those in which the compartments were left intact
and we therefore combined the results from all experiments. We also regularly observed periods of
independent activity on each muscle region in these experiments. In addition to these regions, we also
recorded the activity of the hip flexor muscle iliopsoas. This muscle is reliably activated in this
preparation and defines the basic hip flexion phase of evoked rhythms (Klein et al. 2010). Activity was
amplified near the preparation (10X, A/M Systems) before being amplified and filtered (1000X, 10-1000Hz bandpass A/M Systems), then collected (2500Hz) and saved to disk for offline analyses using
LabView.

Rhythmic activity was evoked by application of a combination of 5-HT (5-9μM) and NMDA (5-9μM), by
stimulation of sacrocaudal afferents by cauda equina stimulation (2-6 Hz, usually 6 Hz; 90 – 300μA;
250μs; 20-30s), or by stimulation of the S2 or L5 dorsal roots (2-6 Hz, usually 6 Hz; 20-150μA; 250μs; 20-30s). Intensity of stimulation (either pharmacological or electrical) was varied until reliable rhythmic
activity was obtained on all recorded muscles. We used stimulation intensities as low as possible to
maintain a stable rhythm. With stronger stimulation, the evoked rhythms often became unorganized or
resulted in tonic activity. For 5-HT/NMDA these lower concentrations often required 5-10 minutes from
the start of wash in until stable patterns were observed. In 5 preparations, we examined the rhythms
evoked by stimulation of cauda equina with and without the conus medullaris included in the
stimulating electrode and found that the rhythms in both cases were very similar to one another.
Stimulation artifact was removed from the recordings in offline processing.
Onsets of iliopsoas were identified manually using custom software in Matlab and were used to define cycles. The activity of each muscle on a given cycle was then rectified and resampled so as to have the same duration as the average cycle for that animal. Each cycle was then divided into 100 bins of equal phase (from onset of iliopsoas in one cycle to onset of iliopsoas in the next cycle) and the activity of the muscle was integrated for each bin. We then characterized the activity of a muscle on a given cycle by calculating the mean phase using circular statistics. (Berens 2009; Klein et al. 2010; Mardia 1972). The mean phase characterizes the part of the cycle in which the activity of a muscle is concentrated. We did not attempt to define onsets and terminations of individual muscle bursts other than for iliopsoas in these analyses since the activity in the different compartments of BFp was often quite sparse and variable (see Figures 5, 6, 7) making robust identification of these times difficult. The mean phase is more robust with respect to this weak activity and we have therefore found it to be a useful and objective measure of the relationship of the muscle activation to the rhythm.

To determine whether the mean phase of a muscle was well defined for a particular burst, we examined the mean resultant length (R value) of the muscle activity for each cycle. The mean resultant length of a muscle reflects the dispersion of muscle 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 a particular R value was significant for a particular cycle, we performed a bootstrap statistical test. The activity of the muscle on the cycle was randomly shuffled across bins and the resulting R value was calculated. This process was repeated 1000 times to create a distribution of R values which would be expected if there were no relation between the activity of the muscle and the rhythm. If the R value that was actually observed was extreme with respect to this distribution (greater than 95% of the distribution), we concluded that the muscle was modulated during that particular cycle of the evoked rhythm. Only cycles for which a muscle was significantly modulated according to this analysis were used in subsequent analyses.

There could be variability in the exact phase of the rhythm in which BFp was activated, both from one cycle to the next in the same animal (Figure 2A, B, C) and from one animal to the next in the same stimulus condition (Figures 3, 5, 6, 7). Because we were mainly interested in the specificity of activation in different regions of BFp, we focused our analyses on the differences between phases recorded for each region. In order to assess whether two regions of BFp were activated differentially for an animal, we first found cycles for which both regions were significantly modulated, as determined by the mean resultant length described above, and calculated the phase difference for each individual cycle. Only
cases for which there were at least 6 such cycles were used for subsequent analyses. We then examined whether the distribution of these phase differences was significantly different from zero, using the M-test for circular statistics (analogous to a one sample t-test; p < 0.05) (Berens 2009). We used a similar analysis to assess whether two regions of BFp were activated differentially across all animals in a particular stimulus condition. We first calculated the mean phase difference between the two regions for each animal. We then examined whether the distribution of these mean phase differences across animals was significantly different from zero, again using the M-test (p < 0.05). To assess whether there was a difference in the distributions of mean phase differences between the rhythms evoked by different stimulation conditions, we used the Watson-Williams test (p < 0.05) (Berens 2009). For post-hoc comparisons we adjusted the significance level by dividing by the number of comparisons (i.e. Bonferroni correction).

Results

Subregions of BFp are activated differentially by the neonatal spinal pattern generator

An example of the activity in the different regions of BFp produced in a 5-HT/NMDA evoked rhythm is shown in Figure 1B. In this example, hindlimb afferent feedback was intact. As can be seen in the figure, all regions of BFp were activated in extension and were out of phase with the hip flexor iliopsoas, consistent with previous descriptions of the rhythms evoked by 5-HT alone (Kiehn and Kjaerulff 1996). However, closer inspection shows that there were systematic differences in the portion of the extension phase at which each region of BFp was activated. In particular, there was a sequential activation of different regions of BFp, with rostral regions activated earlier than more caudal regions. Figure 2A shows the distribution of mean phases for all cycles in the animal shown in Figure 1B. There was a clear sequence of activation across the different regions of BFp, with the distribution of mean phases significantly different between each region of BFp (p < 0.05). Similar patterns were observed in deafferented animals, as illustrated in a different animal in Figure 2B. In this animal the distribution of mean phases was also significantly different between each BFp region (p < 0.05). In Figure 2C we show the activation phase on individual cycles for the rostral and middle regions of BFp from the animal shown in Figure 2B, showing that despite the overlapping distributions of mean phases shown in Figure 2B, this sequential activation was observed consistently across cycles. As can be seen in the figure, in all but 3 cycles the rostral region of BFp was activated before the middle region even though each region
could be activated in a range of phases throughout the cycle. Note also that although the examples in Figure 2A,B might suggest that the distribution of phases was less variable in animals with afferents intact, this was not consistently observed across animals. The variability of mean phases, as measured by the mean resultant length of the distribution, was not significantly different between afferented and deafferented animals for any of the muscles recorded here (p > 0.05 for all comparisons), consistent with what we observed previously for a different set of muscles (Klein et al. 2010).

Figure 3A summarizes the patterns observed for all animals in the rhythms evoked by 5-HT/NMDA with afferents intact. The pattern observed for each individual animal is indicated by a connected set of lines. As seen in the figure, the large majority of animals showed a sequential activation of the different regions of BFp. The mean phase of the rostral region of BFp was significantly earlier in the cycle than the middle region of BFp in 11/11 animals, and the caudal region of BFp was significantly later than both the rostral and middle regions in 11/11 animals (p < 0.05). In no case was the rostral region significantly later than either the middle or caudal regions nor was the caudal region ever significantly earlier than the rostral or middle regions (p > 0.05). Combined across animals, the mean phase of activation for iliopsoas was 56.38 ± 11.80 (in degrees, mean ± SD), rostral BFp was 239.34 ± 22.64, middle BFp was 270.68 ± 21.07, and caudal BFp 304.15 ± 23.37. Considering only the activation of different BFp regions (excluding iliopsoas), there was a significant difference in the mean phases when combined across animals (Watson-Williams test, p < 0.05). Post hoc tests showed that each region was activated at a distinct portion of the evoked rhythm (Watson-Williams test). Because there was variability between animals as to the exact phase at which each portion of BFp was activated (see Figure 3A), we further evaluated the specificity of BFp activation by calculating the phase difference between each region of BFp (Figure 3C; rostral vs. middle, rostral vs. caudal, middle vs. caudal) for each animal, then examining whether these differences were significantly different than zero (Figure 3C). We found that for 5-HT/NMDA evoked rhythms, there was a significant nonzero phase difference between each region of BFp (p < 0.05).

Similar patterns were evoked by 5-HT/NMDA following deafferentation (Figure 3B, 3C), with the rostral region of BFp significantly earlier than middle regions in 5/6 animals and the middle region earlier than the caudal region in 5/6 animals (p < 0.05). Combined across animals, the mean activation phase in deafferented animals was 58.46 ± 6.63 for iliopsoas, 238.16 ± 12.26 for rostral, 257.18 ± 7.51 for middle, and 297.54 ± 17.38 for caudal BFp. There was a significant difference between regions of BFp in deafferented preparations (p < 0.05) and post hoc tests revealed that the phase of each region was
significantly different from the other (Watson-Williams test). Similarly, the phase differences between each region of BFp were significantly different from zero (p < 0.05, Figure 3C). When comparing deafferented and afferented groups, we found no significant difference in the mean phase of activation for each region of BFp (p > 0.05 for each comparison) and the phase delays between each region of BFp were also not significantly different (p > 0.05; Figure 3C). These results show that neonatal spinal central pattern generators are able recruit regions of a single muscle with a high degree of specificity, consistently activating BFp in a rostral to caudal sequence during the rhythms evoked by 5-HT/NMDA.

As mentioned previously (see Methods) we examined rhythms using the lowest stimulation intensity possible to evoke a stable rhythm. Although it was not explored systematically in these experiments, we often noted that as the intensity of stimulation was increased, the relationships between different regions of BFp could subtly change. An example is shown in Figure 4, in which the clear sequential activation of BFp seen when the rhythm was evoked with 5.5/5.5uM 5-HT/NMDA (Figure 4A) became less pronounced when the rhythm was evoked with a concentration of 6/6uM (Figure 4B) although there were still significant differences in the timing of each region of BFp (p< 0.05 between each region of BFp in both low and high drug concentration cases). Note also that the frequency of the rhythm increased in the higher drug concentration. It is therefore unclear from these observations whether the observed changes in phasing are due to the change in concentration or to the change in frequency. Although these issues were not explored systematically here, these observations suggest that even within the rhythms evoked by 5-HT/NMDA, the precise timing between the activations of different regions of BFp could be altered.

Specificity of BFp activation is not stereotyped

The results in Figure 3 show the consistent sequential activation of different regions of BFp from rostral to caudal during 5-HT/NMDA evoked rhythms. As has been described previously, however, rhythms can be evoked in this preparation in several different ways and the patterns which are evoked are not identical. In particular, the activation of muscles evoked from cauda equina (CE) stimulation differs substantially from the patterns evoked by 5-HT/NMDA (Klein et al. 2010). We therefore examined the activity of BFp during rhythms evoked by stimulation of the cauda equina.

An example of a CE evoked rhythm is shown in Figure 5A,B for a deafferented animal. As can be seen in the figure, the rostral and middle regions of BFp were both activated during extension, similar to the
rhythms evoked by 5-HT/NMDA. However, the caudal region of BFp was activated during flexion, in contrast to the mainly extensor related activity in caudal BFp observed in 5-HT/NMDA. In this animal, the distribution of mean phases (Figure 5B) for caudal BFp was significantly different from that of both middle and rostral BFp (p<0.05), but was not different from that of iliopsoas (p > 0.05). As is also illustrated in the figure and as we have shown previously, muscle activity in CE evoked rhythms was more variable when compared to rhythms evoked by 5-HT/NMDA (Klein et al. 2010). Examination of the activity in Figure 5A, however, further suggests that caudal BFp was activated as part of flexion in this CE evoked rhythm. In particular, on the fourth cycle when the flexor burst in iliopsoas was reduced dramatically, the burst in caudal BFp was similarly reduced. Figure 5C summarizes the CE evoked patterns for all animals, illustrating the consistency of flexor related activity in caudal BFp across animals. Note that the phase on the y-axis wraps around as a circle, so that values near the top of the y-axis are similar to values near the bottom. In 6/12 animals rostral and middle regions of BFp were active in significantly (p < 0.05) different portions of the cycle, in 7/9 animals the rostral and caudal regions of BFp were active in different portions, and in 3/6 animals the middle and caudal regions were active in different portions (the difference in total numbers of cases for each comparison is due to the fact that we required at least 6 cycles for which both regions of BFp were significantly tuned in order to make each comparison, see Methods). Notably, in 5/9 animals, there was no significant difference (p > 0.05) between the activation of iliopsoas and the caudal region of BFp. In contrast, iliopsoas activation was significantly different from both the rostral and middle regions of BFp in 13/13 and 12/12 cases, respectively (p < 0.05). These observations further emphasize the flexor related activity of BFp in CE evoked rhythms. We also note that although 6/12 animals had significantly different activation phases between rostral and middle BFp (p < 0.05), in 4 of those 6 cases the middle region of BFp was activated before rostral BFp. This is in contrast to the consistent rostral to caudal sequence observed in 5-HT/NMDA evoked rhythms.

Similar results were obtained when combining data across animals. The mean phase of activation during CE evoked rhythms for iliopsoas was 63.84 ± 13.75, rostral BFp 248.38 ± 27.18, middle BFp 261.58 ± 32.19, and caudal BFp 60.90 ± 53.65. There was a significant difference between the activation phases of different regions of BFp (p < 0.05). Posthoc tests showed that the caudal region of BFp activation phase was significantly different than both the middle and rostral regions of BFp (p<0.05). Middle and rostral regions of BFp were not significantly different from one another (p > 0.05). Similarly, the phase differences between rostral and caudal BFp and between middle and caudal BFp were significantly different from zero (p < 0.05) but the differences between rostral and middle BFp were not
Comparing 5-HT/NMDA and CE stimulation evoked rhythms directly, we found no significant difference in the phase of activation for iliopsoas, rostral BFp, or middle BFp (p > 0.05) but a significant difference between the activation of caudal BFp in the two stimulation conditions (p < 0.05). Note also that these differences between stimulus conditions were observed within individual animals, consistent with our previous work considering other muscles (Klein et al. 2010). In particular, of the 4 animals examined here with both 5-HT/NMDA and CE evoked rhythms, in 3 animals 5-HT/NMDA evoked the typical rostral to caudal activation sequence in BFp and CE stimulation evoked the typical flexor related activation in caudal BFp with extensor activation of middle and rostral BFp. In the remaining animal, 5-HT/NMDA evoked a rostral to caudal sequence but caudal CE was active at the transition between flexion and extension. Taken together, these observations show that the rostral to caudal pattern of BFp activation seen in 5-HT/NMDA evoked rhythms was altered in the rhythms evoked by CE stimulation. In particular, in most cases CE evoked rhythms consisted of flexor related activity in caudal BFp. These results show that not only is the central pattern generator highly specific at birth, but that this specificity is also flexible.

*Patterns of biceps activation in rhythms evoked by L5 dorsal root stimulation*

We examined these same issues of specificity and flexibility further by characterizing the muscle activation patterns in BFp during rhythms evoked by stimulation of individual dorsal roots (L5 or S2). In general, L5 dorsal root stimulation evoked rhythms which were roughly similar to those evoked by 5-HT/NMDA, in that all regions of BFp were activated during extension, as can be seen in the example of Figure 6A. However, there were also subtle differences between the patterns evoked by L5 stimulation and those evoked by 5-HT/NMDA. For instance, in the example shown in Figure 6A, the sequential activation of BFp regions was actually reversed as compared to 5-HT/NMDA patterns, with the middle region earlier than the rostral region and the caudal region earlier than the middle region. Such a caudal to rostral progression of activity was never observed in 5-HT/NMDA rhythms.

Figure 6B,D shows the summary for all animals in which a rhythm was produced by L5 stimulation. In all animals, each region of BFp was activated during extension, but there was clear differential activation of the separate regions of BFp in these rhythms. In 4 of the 5 rhythms evoked by contralateral L5 DR stimulation (Figure 6B), the activation phase of each region of BFp was different from one another (p < 0.05), again demonstrating the specificity of activation within BFp. However, the rostral to caudal sequence of activation observed in 5-HT/NMDA evoked rhythms was not consistently observed in these L5 DR evoked rhythms. In particular, we observed some cases of the reversed sequence of activation,
with caudal regions of BFp activated before rostral ones (0/4 cases with middle before rostral, 1/4 cases with caudal before rostral, 2/4 cases with caudal before middle).

Similar patterns were observed for the 4 animals in which rhythms were evoked by ipsilateral L5 DR stimulation, in which all regions of BFp were active during extension (Figure 6C,E). We also observed cases of rhythms with rostral regions before caudal regions (1/4 cases with rostral before middle, 2/4 cases with rostral before caudal, and 1/4 cases with middle before caudal) as well as patterns with caudal regions before more rostral ones (1/4 cases with middle before rostral, 1/4 cases caudal before rostral, and 1/4 cases caudal before middle). Thus, stimulation of either ipsilateral or contralateral L5 dorsal roots evoked rhythms with differential activation of subregions of BFp. However, this differential activation was not as consistent across animals as that seen in 5-HT/NMDA or CE evoked rhythms.

**Patterns of biceps activation in rhythms evoked by S2 dorsal root stimulation**

We also examined the activation of BFp regions during the rhythms evoked by S2 DR stimulation. Figure 7A shows an example of a rhythm evoked by contralateral S2 DR stimulation. As indicated in the figure, most cases of S2 DR stimulation evoked rhythms in which all regions of BFp were activated during extension, similar to the patterns observed in 5-HT/NMDA and L5 DR stimulation evoked rhythms. As can also be seen in the figure, however, caudal BFp had some activity also during the flexion phase of the rhythm. Such variable activation was often observed in caudal BFp during S2 DR evoked rhythms (see below).

In Figure 7B,C we summarize the rhythms evoked by S2 DR stimulation across all animals. It is interesting to note that in contrast to 5-HT/NMDA and L5 DR stimulation evoked rhythms, there were a few animals (1 animal for contralateral S2, 1 animal for ipsilateral S2) for which caudal BFp was activated during flexion (Figure 7B,C), similar to the rhythms evoked by CE stimulation. Similar to the rhythms evoked by L5 DR stimulation, however, regions of BFp were typically differentially activated even when they were all active during extension. For rhythms evoked by ipsilateral S2 DR stimulation, we observed significantly different activation of rostral and middle BFp in 3/9 cases, of rostral and caudal BFp in 4/9 cases, and middle and caudal BFp in 5/8 cases (p < 0.05). In most cases, activation progressed from rostral to caudal, similar to that observed in 5-HT/NMDA (3/3 cases with rostral before middle BFp, 3/4 cases with rostral before caudal, and 3/5 with middle before caudal). For the rhythms evoked by contralateral S2 DR stimulation, differential activation of BFp was observed, but it appeared to be less common than seen in other rhythms. In 1 of 5 cases, rostral BFp was active before middle BFp, in 2/4
cases rostral was different than caudal BFp (1 case rostral before caudal, 1 case caudal before rostral),
while in 0/2 cases was the activation of middle BFp different from caudal BFp. Taking the results of the
last two sections together, they show that there was typically a differential activation of regions of BFp
in the rhythms evoked by L5 and S2 DR stimulation. Although regions of BFp were generally active
during extension, there were occasionally rhythms evoked by S2 DR stimulation in which caudal BFp was
active during flexion. Further, even when all regions of BFp were active during extension, the particular
pattern of differential activation was not stereotyped, with both rostral to caudal sequences of
activation and caudal to rostral sequences observed. We also note that although L5 and S2 DR
stimulation evoked a range of activity patterns in BFp, in all animals in which 5-HT/NMDA evoked
rhythms were also measured, 5-HT/NMDA consistently evoked patterns with a rostral to caudal
sequential activation of BFp (4/4 animals). In these animals, cases of both rostral to caudal and caudal
to rostral progression within BFp could be observed from DR stimulation. These results reinforce the
findings of a flexible differential control of regions of BFp described above for the rhythms evoked by 5-
HT/NMDA and CE stimulation.

Differential reliability of modulation in different regions of BFp

The examples of rhythms shown in previous sections, especially for S2 and CE evoked rhythms, suggest
that regions of BFp differed in how consistently they were modulated in evoked rhythms. We examined
whether this was the case by comparing the percent of significantly modulated cycles for each region of
BFp. Note that iliopsoas was not included in these analyses since the presence of rhythmic iliopsoas
was an inclusion criterion for considering any rhythms. For the rhythms evoked by 5-HT/NMDA
(afferents intact or deafferented), by L5 DR stimulation (ipsilateral or contralateral), or by ipsilateral S2
DR stimulation, there was no significant difference between these percentages (Kruskal-Wallis test, p >
0.05; rostral, middle, caudal BFp for 5-HT/NMDA intact: 90 ± 10, 91 ± 12, 80 ± 16; for 5-HT/NMDA
deafferented: 72 ± 39, 84 ± 17, 79 ± 21; ipsi L5: 69 ± 33, 83 ± 24, 67 ± 36; contra L5: 88 ± 12, 93 ± 4
86 ± 7; ipsi S2: 86 ± 10, 86 ± 12, 67 ± 21). However, for the rhythms evoked by stimulation of CE and
contralateral S2 DR there was a significant difference between the percent of significantly modulated
cycles across regions of BFp (Kruskal-Wallis test, p < 0.05; rostral, middle, caudal BFp for CE: 89 ± 12, 65
± 20, 42 ± 25; contra S2: 90 ± 12, 72 ± 31, 51 ± 16). In both cases, the caudal regions of BFp were less
likely to be significantly modulated than more rostral regions. It therefore appeared that the caudal
region of BFp which showed the most flexibility in its activation phase across stimulus conditions (often
shifting from extension to flexion), was also the region which was most inconsistently modulated within a particular stimulus conditions (showing significant modulation less often than other regions of BFp).

Discussion

The present study demonstrated the high degree of specificity of central pattern generation in the spinal cord at birth. We demonstrated a differential activation of regions within biceps femoris during rhythms evoked by 5-HT/NMDA, with a consistent activation sequence from rostral regions to caudal regions within extension. Moreover, this differential activation was not stereotyped but was altered depending on the stimulation condition evoking the rhythm. Most dramatically, in CE evoked rhythms and a few S2 evoked rhythms, caudal BFp was typically activated during flexion. There was also evidence for differential activation of BFp regions in the rhythms evoked by L5 and S2, although these patterns were variable: in some cases a rostral to caudal activation sequence was observed, in other cases the sequence was reversed, progressing from caudal to rostral regions of BFp. These results demonstrate that pattern generators within the spinal cord at birth are both highly specific, in that they can differentially activate distinct portions of a single muscle, and that this specificity is flexible, in that the differential activation can be altered across stimulation conditions.

Specificity of pattern generation by spinal motor systems at birth

The main result of this study was that spinal pattern generators at birth are capable of differential control of subregions of a single muscle. Previous work has demonstrated that rhythmic patterns produced in the isolated neonatal spinal cord are not a simple flexion/extension alternation, but that many muscles are active in only a portion of flexion or extension or across their boundary (Hayes et al. 2009; Kiehn and Kjaerulf 1996; Klein et al. 2010). Similarly, although rhythms produced following acute spinalization in adult cats initially consist of a relatively simple flexion/extension alternation, a more specific control can be restored following rehabilitation training (Pearson and Rossignol 1991) or increases in spinal excitability (Dubuc et al. 1986) (Pearson and Rossignol 1991). The present study extends that work to show that the specificity of muscle activation can be observed within different regions of a single muscle, even when the spinal cord is isolated from the rest of the nervous system in neonates. This intramuscular specificity is similar to what has been observed for complex muscles in intact adults during behaviors such as locomotion and responses to postural perturbations (Chanaud
BFp is an especially complex and mechanically heterogeneous muscle (Carrasco and English 1999; Chanaud and Macpherson 1991; Chanaud et al. 1991a). As described above, because of the broad insertion of BFp across the extent of the tibia, the mechanical action of rostral regions would be expected to differ from that of caudal regions. In particular, rostral BFp has a predominant hip extensor action while caudal BFp has an additional knee flexion action (Greene 1935). The sequential activation of BFp from rostral to caudal observed in 5-HT/NMDA evoked rhythms can be related to these different mechanical actions, with early activation of rostral BFp contributing mainly to forward propulsion and delayed activation of caudal BFp contributing to the lift of the limb at the end of extension in preparation for limb protraction. The switch of caudal BFp to flexor related activation observed during CE stimulation evoked rhythms (and some S2 DR evoked rhythms) might prolong knee extension, potentially increasing the duration of propulsion which would be halted by flexion across both hip and knee joints due to activation of iliopsoas and caudal BFp. It is interesting to note that semitendinosus (ST), a muscle with hip extensor and knee flexor actions qualitatively similar to those of caudal BFp, shifts from extensor activity in 5-HT/NMDA evoked rhythms to flexor activity in CE evoked rhythms (Klein et al. 2010), similar to the shifts observed here for caudal BFp. Further, both caudal BFp and ST are activated during extension during L5 DR evoked rhythms. These two muscles thus appear to be activated in parallel in the rat as they are in the cat (Pratt et al. 1991; Rossignol 1996), although experiments recording the activity of these two muscles/muscle regions simultaneously will be necessary to determine the exact relationship between their recruitment during rhythms.

As has been discussed elsewhere, the heterogeneity of mechanical actions within complex muscles such as BFp raises the question of whether such muscles should be considered as a single functional unit (Carrasco and English 1999; Chanaud et al. 1991b; Windhorst et al. 1989) (Loeb 1990). The distinct innervation territories observed in cat biceps femoris have been suggested to comprise functionally separable neuromuscular compartments which might be accessed by the nervous system as distinct control units (Carrasco and English 1999; Chanaud et al. 1991a). Note that although the recordings described here were taken from regions of BFp roughly defined by each nerve branch, we have not attempted to define distinct neuromuscular compartments. While in some cases such neuromuscular compartments appear to have distinct patterns of activation with all motor units within a compartment activated similarly (Pratt and Loeb 1991), in the cat BF does not appear to be strictly segregated between
neuromuscular compartments (Chanaud and Macpherson 1991), suggesting instead a more individuated recruitment of motor units through the extent of BFp. The sequential activation of BFp observed in the present experiments suggests a similar individuated control by neonatal pattern generators in the rat, although recordings with more spatial resolution are required to establish this definitively. Given these observations of intramuscular specificity, it is unclear whether the fact that motor units are located in the same muscle is more predictive of their activation than the fact that the units have similar mechanical actions. In this context, the intramuscular specificity demonstrated here could be considered as simply a higher degree of the same type of specificity observed in the activation of different muscles.

Finally, it is interesting to note that the intramuscular specificity of activation described in this study is produced in the context of a generally variable motor pattern (Cazalets et al. 1990; Klein et al. 2010), especially when compared to the adult. As we have discussed elsewhere (Klein et al. 2010), such variability might be expressed during development while the nervous system is identifying the properties of the musculoskeletal system, using this variability to explore the potential consequences of different motor commands (Petersson et al. 2003; Tumer and Brainard 2007). This variability might be advantageous even when neural systems have already obtained (either through experience or genetics) fairly specific knowledge of muscle properties, allowing for further refinement or for identifying effective coordination patterns. In this context, the specificity of muscle activation within a generally crude motor pattern suggested by the present study could be considered as reflecting a development transient in which the spinal cord is capable of exploiting the knowledge that it already has (the mechanical actions of different parts of BFp) while still exploring to refine this knowledge or to use it more adaptively.

Flexibility in spinal pattern generators

These results extend our previous demonstrations of a high degree of flexibility in the motor patterns produced by spinal systems (Klein et al. 2010). We found here several distinct patterns of activation across the three regions of BFp. 5-HT/NMDA evoked a robust rostral to caudal sequence of activation, with all regions of BFp active during extension. CE stimulation typically evoked a pattern with activation of caudal BFp shifted to flexion. L5 and S2 DR stimulation usually evoked patterns in which all regions of BFp were active in extension, but the rostral to caudal sequence observed in 5-HT/NMDA was not
consistently observed. Moreover, S2 dorsal root stimulation occasionally evoked rhythms similar to CE
evoked rhythms, with flexor related activity in caudal BFp. Such flexibility was not dependent on
afferent feedback since these different patterns were observed in deafferented preparations. This
flexibility demonstrates the richness of pattern generation within spinal motor systems at birth. Far
from producing a stereotyped motor pattern, these results show that spinal networks can produce a
range of motor patterns, potentially adapting the activation of motor units distributed throughout the
musculature according to the manner in which spinal systems are activated.

As we have discussed previously (Klein et al. 2010), it is unclear whether the ability of spinal systems to
generate multiple patterns reflects the action of a single network which is altered by stimulation
condition or the action of multiple networks. Given the consistent activation of muscles such as
iliopsoas and semimembranosus across all these rhythms (Klein et al. 2010), it seems likely that the
networks underlying these rhythms are at least partially overlapping. The results observed here could
also be considered in the context of central pattern generator models consisting of separable rhythm
and pattern generating networks (Burke et al. 2001) (Rybak et al. 2006) (Rybak et al. 2006). In this
context, one could consider that the evoked pattern in each stimulus condition relied on a common
rhythm generating network, specifying the basic alternation of muscles such as iliopsoas and
semimembranosus, but that the pattern generating network changed across stimulus condition so that
the activity of other muscles (semitendinosus, rectus femoris, BFp) was altered.

It is also interesting that caudal BFp was both the most flexible region of BFp, in that it was the most
likely region of BFp to show large changes in its activation phase across stimulation conditions, and was
also the most variable, in that it was the least likely to show significant modulation within a stimulus
condition, at least in the rhythms evoked by CE and contralateral S2 stimulation. In our previous work
(Klein et al. 2010), we have similarly shown that RF and ST, which also shift their activation phases
between flexion and extension in 5-HT/NMDA and CE evoked rhythms, are also less likely to be
significantly modulated. In contrast, muscles such as iliopsoas, SM, AM, and the rostral and middle
regions of BFp did not change their activation phases as dramatically across stimulation conditions and
were more reliably modulated. One possible explanation for the lower modulation of these more
complex muscles could be that they receive synaptic drive during both flexion and extension. Although
such mixed activation during both flexion and extension could reflect the immature state of neonatal
spinal pattern generators, experiments examining the recruitment of motor neurons innervating such
bifunctional muscles in adult cats have shown that they can receive such mixed synaptic drive (Perret
Such a mixed drive, if confirmed for the neonate, would therefore lend further support to the idea that spinal pattern generators immediately at birth are similar to those in adults.

**Implications for neural control strategies**

The specificity of muscle activation demonstrated here also raises the question of the level of control exerted by the nervous system (Tresch and Jarc 2009). At the extreme of specificity, the nervous system might consider the musculoskeletal system as a collection of motor units, with each unit having a potentially unique contribution to behavior. In this scenario, the degrees of freedom controlled by the nervous system would be equal to the number of motor units within the musculoskeletal system. Such a control scheme, although complex, would afford the nervous system a high degree of flexibility and functional specificity. Note that such a control scheme would not necessarily require a detailed motor plan with the activation of each motor unit planned individually. Strategies such as optimal feedback (Todorov 2004), minimum intervention (Valero-Cuevas et al. 2009), and used in uncontrolled manifold analyses (Latash et al. 2007) do not require an explicit plan for each degree of freedom, but only require that their aggregate action achieves behavioral goals. Although these ideas are usually proposed at the level of entire muscles, it would not be surprising to observe a similar level of control produced at the level of motor units. On the other hand, other observations suggest that the nervous system exerts control at the level of groups of motor units either within or across muscles (Berniker et al. 2009; Bizzi et al. 2002; Ting 2007; Tresch and Jarc 2009) (d’Avella et al. 2008) (Giszter et al. 2007). For instance, recent formulations of ‘muscle synergies’ suggest that instead of controlling individual muscles or motor units, the nervous system might control groups of muscles or populations of motor units when producing behaviors (Berniker et al. 2009; Bizzi et al. 2002; Ting 2007; Tresch and Jarc 2009) (Giszter et al. 2007) (d’Avella et al. 2008). These formulations are also related to the ideas of modules and unit burst generators which are often used to explain rhythmic motor control (Grillner 1985; Jordan 1991; Stein 2008). Although such a control scheme could reduce the complexity involved in motor control, it would also reduce the flexibility and functional adaptation of the nervous system (Tresch and Jarc 2009) (Kutch et al. 2008; Valero-Cuevas et al. 2009).

The present demonstration of specificity in intramuscular activation can most straightforwardly be interpreted as supporting the former idea, that the nervous system controls movement with a high
degree of flexibility and precision. The detailed and consistent delays between regions of BFp observed here, along with the range of different patterns in these delays across stimulus conditions, suggest individuation in the control exerted by spinal motor systems. However, it is obviously difficult to make such a conclusion definitively. For instance, the similarity in phase shifts across stimulation conditions for caudal BFp and ST as well as observations such as the apparent ‘flexion deletion’ (Stein 2008) in Figure 5A suggest that these groups of muscles might controlled together in the evoked patterns, consistent with the idea of muscle synergies. Moreover, some of the phase shifts between regions of BFp observed here could be interpreted as ‘time-varying’ muscle synergies (d'Avella and Bizzi 2005; d'Avella et al. 2003), in which coordination across muscles is fixed in both the strength and the timing of activation. It is also important to note that simply observing phase shifts between different regions of BFp does not necessarily reflect differences in the central drive to the motor neurons innervating these regions. Instead, these phase shifts could reflect a differential shaping of a common central drive by intrinsic properties of motor neurons, although the variety of phase shifts observed here is difficult to explain entirely by such a mechanism. In afferent intact preparations, phasic sensory feedback could also help sculpt motor neuron activity patterns. Resolving the observations of individuated control, such as those of the present study, with the observations of coordination across muscles will require further experiments examining both the simultaneous activity across groups of muscles and the organization of neural systems responsible for their activity.

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Figure Legends

Figure 1 (A) shows a schematic indicating the different regions of the biceps femoris which were recorded from. We recorded from three sites along the medial surface of biceps femoris: a rostral region (rostral BFp; rBFp) with insertion near the femur, a caudal region (caudal BFp; cBFp) with an insertion on the tibia toward the ankle, and a middle region (middle BFp; mBFp) intermediate to the other two. Note that none of these recordings were from the anterior head of biceps femoris which has its origin on spinal vertebrae since it is not readily accessible from medial recordings. (B) shows an example the activity on iliopsoas (IP) and the different regions of biceps femoris. In this experiment, the three different regions of BF were partly dissected with a small pin and separated with dissecting pins.
and afferents were intact. As can be seen in the figure, although all regions of BF were active during extension, there was a sequential activation of different regions so that rostral regions preceded more caudal regions.

Figure 2 (A) Distribution of mean phases across all cycles for the animal illustrated in Figure 1B. The phase of the rhythmic cycle is indicated on the x-axis, with 0 and 360 degrees indicating the onset of iliopsoas on two adjacent cycles and the transition between flexion and extension occurring approximately at 180 degrees. (B) shows another example of the distributions of mean phases in a deafferented animal (not the same as in (A)). In (C) we show the mean phase on rostral and middle regions of BFp plotted against one another for all individual cycles for the animal shown in (B). A unity line is superimposed, indicating the position in the plot which would be expected if the two regions had the same mean phase on individual cycles.

Figure 3 Summary of all animals with rhythms evoked by 5-HT/NMDA. (A) shows the mean phase of each muscle for individual animals with afferents intact. Each interconnected line indicates a single animal. In cases where not all regions of BFp had at least 6 significantly modulated cycles, only individual data points are indicated. (B) shows the same information for deafferented animals. (C) summarizes the differences in mean phases between regions of BFp (r/m: rostral vs. middle; r/c: rostral vs. caudal; m/c: middle vs. caudal) for afferented and deafferented animals.

Figure 4 An example of rhythms evoked at varying concentrations of 5-HT/NMDA. In the rhythm evoked by the low concentration of 5-HT/NMDA there was a clear sequential activation of BFp from rostral to caudal. With a slightly higher concentration of 5-HT/NMDA in the same animal (B), the rhythm became more intense and faster, and the delays between different regions of BFp were reduced.

Display gains in (A) and (B) are the same.

Figure 5 Rhythms evoked by cauda equina stimulation. (A) shows an example of a rhythm evoked by cauda equina stimulation. Note that the reduced activation of iliopsoas is accompanied by a reduced activation of caudal BFp on the fourth cycle in the figure. (B) shows the distribution of mean phases for all cycles for the animal shown in (A). (C) summarizes the responses for all individual animals for CE evoked rhythms, showing the consistency of the flexor related activity in caudal BFp. (D) shows the averaged difference in mean phase between each region of BFp for CE evoked rhythms.

Figure 6. Rhythms evoked by L5 DR stimulation. (A) shows an example of a rhythm evoked by contralateral L5 DR stimulation. The mean phases for all rhythms evoked by ipsilateral L5 DR stimulation
are shown in (B); the mean phases for rhythms evoked by contralateral L5 DR stimulation are shown in (C). The difference in mean phases between BFp regions are shown in (D) for ipsilateral L5 DR rhythms and in (E) for contralateral L5 DR evoked rhythms.

Figure 7 Rhythms evoked by S2 DR stimulation. An example of an ipsilateral S2 DR stimulation evoked rhythm is shown in (A). Conventions for the rest of the figure are the same as in Figure 6.

References


A 5-HT/NMDA

mean phase (deg)

afferents intact

rBFp mBFp cBFp

BFp region

deafferented

B

mean phase (deg)

rBFp mBFp cBFp

BFp region

C

phase difference (deg)

regions compared

afferents intact

deafferented

r/m m/c r/c
A 5-HT/NMDA (5.5/5.5 μM)

B 5-HT/NMDA (6/6 μM)

IP

rBFp

mBFp

cBFp

mean phase (degrees)
cauda equina

(A) Waveform

(B) Distribution of phase

(C) Mean phase (degrees) for rBFp, mBFp, cBFp

(D) Phase difference (degrees) for regions compared
A contralateral L5 DR stimulation

IP
rBFp
mBFp
cBFp

1s

B mean phase (deg)
contralateral L5 DR

IP
rBFp
mBFp
cBFp
Contralateral L5 DR

C mean phase (deg)
ipsilateral L5 DR

D phase difference (deg)
regions compared

E phase difference (deg)
regions compared

BFP region
r/m m/c r/c

phase difference (deg)
r/m m/c r/c
regions compared

phase difference (deg)
r/m m/c r/c
regions compared

1s
ipsilateral S2 DR stimulation

B

mean phase (deg)

contralateral S2 DR

D

phase difference (deg)

regions compared

C

mean phase (deg)

ipsilateral S2 DR

E

phase difference (deg)

regions compared

A

contralateral S2 DR