Properties of Rhythmic Activity Generated by the Isolated Spinal Cord of the Neonatal Mouse

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Whelan, Patrick, Agnes Bonnot, and Michael J. O’Donovan. Properties of rhythmic activity generated by the isolated spinal cord of the neonatal mouse. J Neurophysiol 84: 2821–2833, 2000. We examined the ability of the isolated lumbosacral spinal cord of the neonatal mouse (P0–7) to generate rhythmic motor activity under several different conditions. In the absence of electrical or pharmacological stimulation, we recorded several patterns of spontaneous ventral root depolarization and discharge. Spontaneous, alternating discharge between contralateral ventral roots could occur two to three times over a 10-min interval. We also observed other patterns, including left-right synchrony and rhythmic activity restricted to one side of the cord. Trains of stimuli delivered to the lumbar/coccygeal dorsal roots or the sural nerve reliably evoked episodes of rhythmic activity. During these evoked episodes, rhythmic ventral root discharges could occur on one side of the cord or could alternate from side to side. Bath application of a combination of N-methyl-D-aspartate (NMA), serotonin, and dopamine produced rhythmic activity that could last for several hours. Under these conditions, the discharge recorded from the left and right L1–L3 ventral roots alternated. In the L1–L5 segments, the discharge had two peaks in each cycle, coincident with discharge of the ipsilateral and contralateral L1–L5 roots. The L6 ventral root discharge alternated with that recorded from the ipsilateral L1–L3 roots. We established that the drug-induced rhythm was locomotor-like by recording an alternating pattern of discharge between ipsilateral flexor and extensor hindlimb muscle nerves. In addition, by recording simultaneously from ventral roots and muscle nerves, we established that ankle flexor discharge was in phase with ipsilateral L1/L2 ventral root discharge, while extensor discharge was in phase with ipsilateral L6 ventral root discharge. Rhythmic patterns of ventral root discharge were preserved following mid-sagittal section of the spinal cord, demonstrating that reciprocal inhibitory connections between the left and right sides of the cord are not essential for rhythmogenesis in the neonatal mouse cord. Blocking N-methyl-D-aspartate receptors, in both the intact and the hemisected preparation, revealed that these receptors contribute to but are not essential for rhythmogenesis. In contrast, the rhythm was abolished following blockade of kainate/AMPA receptors with 6-cyano-7-nitroquinoxalene-2,3-dione. These findings demonstrate that the isolated mouse spinal cord can produce a variety of coordinated activities, including locomotor-like activity. The ability to study these behaviors under a variety of different conditions offers promise for future studies of rhythmogenic mechanisms in this preparation.

INTRODUCTION

The neural mechanisms underlying mammalian locomotion are poorly understood. During the last few years, the isolated spinal cord of the rat has become a popular model system for studying locomotor networks (Kudo and Yamada 1987; Smith and Feldman 1987). This preparation offers several experimental advantages including easy access to the spinal cord and manipulation of the extracellular environment. Locomotor patterns can be readily elicited in this preparation using drugs or electrical stimulation. In contrast to the work on the neonatal rat, there have been few studies of rhythmic behaviors using the in vitro mouse spinal cord. This is somewhat surprising given that the mouse cord has great potential for genetic manipulations (Cazalets et al. 2000; Funk et al. 1997; Smith et al. 1993). Moreover, it has recently been shown that spinal network activity in the neonatal mouse can be visualized with calcium-sensitive dyes (Bonnot et al. 1998b, 1999) and this should facilitate circuit analysis.

It has been shown that the isolated neonatal mouse spinal cord can generate spontaneous bouts of rhythmic alternating ventral root discharge (Bonnot et al. 1998a). Thus, mechanisms underlying rhythmogenesis can be explored without the use of exogenous drugs. In a few reports, drugs that elicit rhythmicity in the rat have been applied to the mouse spinal cord, but the results have been mixed. Hernandez et al. (1991) reported that drugs that evoked locomotor-like activity in the isolated rat cord were ineffective in the mouse cord. This finding was confirmed by Jiang et al. (1999), who also showed that rhythmic alternating ventral root discharge could be induced in the spinal cords of P5–20 mice using a combination of dopamine, serotonin (5-HT), and N-methyl-D-aspartate (NMDA). On the other hand, it has been reported that 5-HT in isolation can elicit rhythmic alternating activity in P0–3 mice (Nishimaru et al. 2000), using similar concentrations to those used to activate rhythmic activity in the in vitro neonatal rat cord.

In this paper, we have built on and extended previous observations of rhythmic activity in the neonatal mouse spinal cord by characterizing the patterns of spontaneous activity in detail and by defining the pharmacological conditions that can induce reliable locomotor-like activity in the neonatal mouse cord. By recording the underlying subthreshold potentials from ventral roots, an unexpected complexity to the spontaneous rhythmic patterns was revealed. In addition to the alternating discharge reported by Bonnot et al. (1998a), other rhythmic behaviors are expressed including subthreshold oscillations

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and unilateral rhythmic discharge. We also show that electrical stimulation of dorsal roots can elicit rhythmic alternating or unilateral discharge, depending on the stimulus intensity.

In contrast to other modes of activation, application of drugs generally produced a regular rhythm that was sustained for several hours. By examining the discharge from flexor and extensor nerves, we found that the recorded patterns were qualitatively similar to those recorded from the same muscles during walking in the mouse (Fortier et al. 1987) and could be correlated with the discharge from rostral and caudal ventral roots. In the second part of the paper, we examine some of the properties of the network responsible for the drug-induced locomotor activity. We show that each half of the cord contains sufficient circuitry to produce alternating flexor/extensor discharge. Finally, we show that rhythmic activity can be expressed during blockade of N-methyl-D-aspartate (NMDA) receptors but not during blockade of AMPA/kainate receptors.

Some of this work has been published in abstract form (Bonnot et al. 1999; Whelan et al. 1999).

**METHODS**

Experiments were performed on Swiss Webster mice (Taconic Laboratory), 1–8 days old (P0–7). After induction of anesthesia with methoxyflurane, the animals were decapitated and eviscerated. The remaining tissue was placed in a dissecting chamber containing artificial cerebrospinal fluid (ACSF) (concentrations in mM: 128 NaCl, 4 KCl, 1.5 CaCl₂, 1 MgSO₄, 0.5 NaH₂PO₄, 21 NaHCO₃, 30 D-glucose), bubbled with 95% O₂:5% CO₂. Two types of in vitro preparation were used. In the first, the animals were pinned down onto a silicone elastomer (Sylgard) base, a ventral laminectomy was performed, and the cord to the level of S₃. The spinal cord was usually transected at the level of T5–T7 and the dorsal and ventral roots were cut up to T12. The cord was then transected between T5 and T7 and gently removed from the vertebral column to T13, and tissue rostral to T12 was discarded. We then performed a dorsal laminectomy from T₁₃ to S₁₃. The lateral gastrocnemius and soleus (LGS) and/or the medial gastrocnemius (MG) and the common peroneal (CP) nerves were dissected out, and the sciatic nerve was freed from the surrounding tissue (see Fig. 7A). The legs were detached at the pelvis, and the remaining tissue was trimmed away from the preparation. Some muscle tissue was left attached to the preparation to minimize the possibility of damage to the nerves and ventral roots. Following these procedures, the preparation was transferred to the recording chamber and bubbled with ACSF continuously recirculated at room temperature or at 27°C.

**Electrophysiological recordings and stimulation protocols**

Motoneuronal activity was recorded with tight-fitting plastic suction electrodes into which individual ventral roots were drawn. The resultant neurograms were amplified (1,000–5,000 times), filtered (DC to 3 kHz; 100 Hz to 1 or 3 kHz; 0.1 Hz to 3 kHz), digitized (NeuroData), and recorded on videotape for further analysis. We used several methods to evoke rhythmic activity. On occasion, spontaneous activity comprised large depolarizations accompanied by several cycles of rhythmic ventral root discharge. In addition, alternating ventral root discharge could be evoked by electrical stimulation of the cauda equina or lumbar dorsal roots using tight-fitting suction electrodes similar to those used for recording (trains: 4–8 Hz; stimulus duration: 150–500 μs, 20–300 μA; train duration: 10–20 s). Finally, in some preparations, we induced rhythmic activity pharmacologically by bath application of various combinations of the following drugs [5-HT, 10–20 μM; N-methyl-D-aspartate (NMA), 5 μM–10 μM; dopamine, 50–100 μM] (cf. Jiang et al. 1999). On isolated spinal cords, a single dose of the chemicals applied to the bath usually produced alternating activity within 5–10 min. When using preparations with attached nerves, we typically started with the dose used on isolated spinal cords but found that rhythmic activity tended to appear in flexor and extensor nerves only if the dose was increased [5-HT (15–20 μM), NMA (7.5–10 μM), dopamine (75–100 μM)].

**Sectioning of the spinal cord**

In some preparations, the spinal cord was sectioned midsagittally. For this purpose, the cord was transferred to a separate chamber where it was sectioned using a pair of ultra fine clippers (FST, Vancouver, Canada) or a tungsten needle. The ACSF within the sectioning cham-

![FIG. 1. Spontaneous electrical activity recorded from contralateral lumbar (L₁) ventral roots in a P2 mouse showing the variability of subthreshold depolarizations and discharge. A: the recordings show a low-pass filtered record (DC to 10 Hz) of the ventral root activity recorded over a 150-s period. The schematic of the cord to the right illustrates the recording arrangement. Electrical recordings from ventral roots were obtained by using tight-fitting suction electrodes. B: the recordings were high-pass filtered (0.1–1 kHz) to show the discharge associated with the record in A. The gray box demarcates an episode of alternating discharge that has been expanded in the last set of traces to show the individual cycles of discharge. This figure has been enhanced on the web to include a scrolling stripchart (see supplementary materials, http://jn.physiology.org/cgi/content/full/84/6/2821/DC1).
was maintained at the same temperature and with the same concentration of drugs as the recording chamber. In another set of experiments the spinal cord was transected at the L₆–S₁ or S₂ level.

Data analysis

Data analyses were performed off-line and selected sequences were digitized using Labview acquisition software along with Axograph analysis software (DC to 10 Hz or 100–500 Hz) to determine cycle period and phase. If necessary, rectification and smoothing of the burst discharges were performed digitally (moving average over 150–200 points). The cycle period was determined by measuring either the peak activity of the low-pass filtered record (DC or 0.1–10 Hz) or the onset of the smoothed rhythmic discharge (100–500 Hz and digitized at 1 kHz). Phase diagrams were constructed by normalizing the onset and offset of rhythmic discharges from different ventral roots to the cycle period of the discharge obtained from one ventral root or nerve (see diagram in Fig. 7A).

The number of episodes of spontaneous alternating and unilateral discharge were counted over a 10- to 30-min interval. An example of such a series is highlighted in Fig. 1. In experiments where DC potentials were recorded, we compared the peak amplitude of the potentials associated with discharging and nondischarging events. An event was defined by the time the DC potential exceeded 10% of the maximum peak-peak depolarization recorded over a 20- to 30-min period. The amplitude of each event was then normalized to the peak amplitude recorded in each experiment.

Statistical comparisons between experimental conditions were made using a t-test or one-way ANOVA if the data were normally distributed and of equal variance. Otherwise, the data were compared using a Mann-Whitney rank sum test.

RESULTS

In the first part of the paper, we describe the characteristics of rhythmic activity generated by the isolated lumbosacral spinal cord of the neonatal mouse under three different conditions: occurring spontaneously, during stimulation of the lumbar dorsal roots or cauda equina, or in the presence of drugs. One goal of the present work was to establish the extent to which the various rhythms were locomotor-like. For this purpose, we define locomotor-like activity as rhythmic activity in which there is an alternating discharge between left and right lumbar ventral roots and between hindlimb flexor and extensor muscles or muscle nerves. In the absence of muscle nerve recordings, we consider locomotor-like rhythmic activity to be a rhythmic alternation of discharge between the left and right lumbar ventral roots coupled with an ipsilateral alternation between the discharge of the L₁–L₂ and L₆ ventral roots. We define this pattern of activity to be locomotor-like because we will show that discharge of L₁–L₂ ventral roots is synchronous with flexor activity and that of the L₆ with extensor activity.

Spontaneous episodes of rhythmic ventral root activity

GENERAL DESCRIPTION OF ACTIVITY. Spontaneous slow potentials and discharge were recorded from lumbar and sacral ventral roots in the spinal cords isolated from 33 mice. The slow potentials recorded from the ventral roots represent electrotonically recorded potentials from populations of motoneurons. As Fig. 1A shows, over a 20- to 30-min recording period (see also scrolling chart on-line, http://jn.physiology.org/cgi/content/full/84/6/2821/DC1), the amplitude and duration of the spontaneous ventral root depolarizations were highly variable [2.6 ± 1 (SD) episodes/min; n = 3; Fig. 1A]. In a particular segment, the onset of depolarizing events recorded from the left and right ventral roots was generally synchronous, but it could be quite variable across different lumbar roots. The depolarizations could occur in the absence of firing or could be accompanied by a few cycles of discharge (Fig. 1B). Several different patterns of rhythmic activity occurred on the left and right ventral roots including: alternation (Fig. 2A), rhythmic discharge on one root of a segment (Fig. 2B, unilateral segmental rhythmic discharge), and synchronized subthreshold oscillations (Fig. 2C). We recorded from various ventral root and nerve combinations (L₁–L₆, S₁–S₃, LGS and CP) depending on the experiment, but the recordings most often included L₁–L₂ ventral roots.

FIG. 2. The isolated spinal cord of the mouse can produce several patterns of spontaneous rhythmic activity. A: rhythmic patterns of activity recorded from the L₁ and L₆ ventral roots of a P3 mouse. The recordings in A show the high-pass filtered discharge (0.1–1 kHz). B: unilateral discharge on the right L₂ and L₄ roots is followed by unilateral discharge on the left L₂ and L₄ roots (P3 mouse). C: low-pass filtered record (DC to10 Hz) showing high-frequency synchronous oscillations recorded from the L₁ and L₄ ventral roots of a P4 mouse. D: integrated, rectified, and averaged traces obtained from 4 different episodes of rhythmic activity (same preparation illustrated in A). E: expansion of area in the gray box in C.
alternating rhythmic discharge. Episodes of alternating discharge between left and right lumbar ventral roots were present in 69% (18/26) of lumbar cords. These episodes of alternation were usually short, comprising two to six cycles of discharge (Figs. 1 and 2A, Table 1). Alternating episodes of left/right ventral root discharge occurred less frequently than the spontaneous depolarizations and could often be observed in more than one segment (Table 1). Figure 2, A and D, shows that the discharge recorded ipsilaterally from the L6 ventral roots could alternate with the discharge recorded from the L1 root. While spontaneous episodes could be expressed selectively in one or more segments, we did not observe a significant difference (P > 0.1) in the frequency of such activity in different parts of the lumbar cord (Table 1).

We found that the amplitude of the slow potentials associated with alternating discharge was significantly larger than that associated with nonalternating discharge. When the peak amplitude of the slow potentials was normalized to the maximum level recorded over a 20- to 30-min period, the average amplitude for alternating episodes was 0.84 ± 0.11 (n = 3, 18 episodes) and for nonalternating episodes was 0.48 ± 0.20 (n = 3 preparations; means ± SD from 160 episodes). In each of the three preparations where these measurements were done, these differences were significantly different (P < 0.001). Alternating discharge was also recorded from the sacral roots in 12 of 16 spontaneously active preparations. The cycle periods for these alternating episodes were greater than that recorded from lumbar ventral roots (Table 1, P < 0.0001).

unilateral rhythmic discharge. In 89% (23/26) of spontaneously active lumbar preparations, we observed episodes of unilateral rhythmic discharge restricted to one or more segments of the cord. An example of this pattern is shown in Fig. 2B. In this example, during unilateral bursting there was discharge in the L2 and L4 ventral roots, although rhythmic activity was only present in the L2 segment during alternating activity. Again, this illustrates the wide variety of inter-segmental coupling patterns that we observed. As with patterns of alternating discharge, the occurrence of unilateral rhythmic discharge was relatively infrequent (Table 1). We found that the slow depolarizing potentials associated with unilateral bursting had a faster rhythm (P < 0.001) compared with the alternating episodes of discharge and could be divided into three main patterns (Table 1). In the first of these, the unilateral discharge was accompanied by synchronized, subthreshold rhythmic depolarizations on the contralateral side. The second type of activity comprised rhythmic discharge on one side without observable subthreshold activity on the other side. The third type, which was observed in three preparations, was accompanied by subthreshold contralateral depolarizations that alternated with the unilateral discharge.

Rhythmic oscillations synchronized across several segments. We also observed another pattern of activity that comprised relatively fast oscillatory depolarizations of up to 10 or more cycles of activity in 73% of the preparations (24/33). The oscillations were synchronized between the left and right sides of the cord and over many segments (Table 1, Fig. 2C) and in some experiments could produce firing.

Rhythmic alternating ventral root activity induced by stimulation of the lumbar or coccygeal dorsal roots or the sural nerve.

In several different species, sensory stimulation can elicit rhythmic activity (Carter and Smith 1986; Deliagina et al. 1984; Grillner and Zangger 1979; Koshland and Smith 1989; Mortin and Stein 1990; Pearson and Rossignol 1991; Smith et al. 1985). For example, pinching the base of the tail or the perineum is an established method for inducing walking in cats with transections of the spinal cord at T12–T13 (Barbeau and Rossignol 1987). In addition, stimulation of the lumbar dorsal roots or tail pinch in the in vitro preparation of the neonatal rat can elicit alternating rhythmic discharge from sacral or lumbar ventral roots (Lev-Tov et al. 2000; Smith et al. 1988). For these reasons, we performed experiments to establish if sensory stimulation could evoke patterned rhythmic output in the neonatal mouse cord.

Stimulation of the cauda equina. In preliminary experiments, we used an isolated spinal cord preparation that included the tail and its associated musculature. If the base of the tail was mechanically or electrically stimulated, the preparation generated rhythmic alternating discharge that could be recorded from the left and right ventral roots (data not shown). In later experiments, we stimulated the cauda equina (containing coccygeal dorsal roots), which produced a qualitatively similar pattern of alternating discharge that could be recorded from lumbar and sacral roots (15/19 animals; P0–6; Fig. 3). The rhythm was characterized by discharge of the sacral and the L4–L5 lumbar roots (cycle period: 1.57 ± 0.43 s, phase: 0.49 ± 0.10, 154 cycles, n = 7 preparations). In the youngest animals, (P0; 4/19) stimulation of the cauda equina usually evoked an irregular rhythm that was poorly coordinated between the left and right sides (data not shown).

<table>
<thead>
<tr>
<th>Type of Activity</th>
<th>Ventral Roots or Nerves</th>
<th>No. of Preparations</th>
<th>Episodes/Min</th>
<th>Cycle Period, s</th>
<th>Phase (Side to Side)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternating</td>
<td>Lumbar (L1–L3)</td>
<td>12</td>
<td>0.25 ± 0.19</td>
<td>0.94 ± 0.24</td>
<td>0.50 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>Lumbar (L4–L6)</td>
<td>5</td>
<td>0.14 ± 0.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sacral</td>
<td>5</td>
<td>0.22 ± 0.16</td>
<td>1.84 ± 0.77</td>
<td>0.46 ± 0.12</td>
</tr>
<tr>
<td>Unilateral rhythmic discharge</td>
<td>Lumbar (L1–L6)</td>
<td>14</td>
<td>0.42 ± 0.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sacral</td>
<td>3</td>
<td>0.23 ± 0.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contralateral synchronized subthreshold</td>
<td>Lumbar (L1–L4)</td>
<td>10</td>
<td>0.72 ± 0.28</td>
<td>0.03 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>Contralateral no subthreshold</td>
<td>Lumbar (L1–L4)</td>
<td>10</td>
<td>0.63 ± 0.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contralateral alternating subthreshold</td>
<td>Lumbar (L1–L4)</td>
<td>3</td>
<td>0.61 ± 0.18</td>
<td>0.49 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>Subthreshold oscillations</td>
<td>Lumbar/Sacral</td>
<td>8</td>
<td>0.28 ± 0.11</td>
<td>−0.01 ± 0.09</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD.
We next established if stimulation of lumbar afferents could also evoke rhythmic activity. We found that electrical alternating discharge could be recorded between the left and right sacral (S₁–S₄) roots in all three preparations (Fig. 3B). We also found that the isolated sacral segments were capable of generating episodes of spontaneous alternation (data not shown). These findings indicate that rhythmogenic circuitry capable of generating alternating discharge between the left and right ventral roots exists in the sacral segments.

Stimulation of lumbar dorsal roots or the sural nerve. We next established if stimulation of lumbar afferents could also evoke rhythmic activity. We found that electrical stimulation of lumbar dorsal roots produced alternating ventral root discharge in the lumbar roots of 10/13 preparations tested (4 Hz for 10 s; Fig. 4A). The rhythm recorded from these roots was superimposed on a background of tonic discharge (see Fig. 4A, DC records). Rhythmic alternating discharge was induced in contralateral lumbar ventral roots when the dorsal root stimulus averaged 1.8 times the threshold for the monosynaptic reflex recorded from an ipsilateral ventral root (range 1.15–3.6 × T, n = 3, L₁–L₃ dorsal roots stimulated, Fig. 4, B and C). At stimulation intensities below this level, rhythmic activity was generally induced on one side of the cord. The cycle period and phase of the evoked rhythmic activity were respectively: 0.82 s ± 0.16 and 0.50 ± 0.10 (L₁–L₃ ventral roots, n = 6 preparations). The evoked rhythmic discharge generally coincided with the stimulus train, although one to two cycles and a tonic discharge sometimes outlasted the train. When the stimulus intensity was increased beyond the threshold for alternating discharge, the activity became progressively more disorganized and eventually became tonic (data not shown). Rhythmic activity could also be recorded from caudally located ventral roots (L₄–L₆). We also observed periods of alternating ipsilateral discharge between the L₁ and L₄ ventral roots. However, the pattern of rhythmic discharge from L₆ was weak and the phase relations between the two sets of roots could only
be clearly discerned after low-pass filtering. In two preparations, we applied the stimulus train to the sural nerve and recorded alternating discharge from the L1/L2 ventral roots in both cases. In one of these preparations, we compared the rhythmic activity recorded from the lumbar ventral roots and the flexor CP muscle and found that the CP discharge was in phase with the ipsilateral L1 burst (Fig. 4D).

Rhythmic activity induced pharmacologically

Patterns of rhythmic activity produced by combinations of NMA, 5-HT, and dopamine. In our initial experiments, we used a combination of NMA (5 μM), 5-HT (10 μM), and dopamine (50 μM) to induce rhythmic activity as first described by Jiang et al. (1999). We found that alternating left/right ventral root discharge appeared approximately 5–10 min after addition of the drugs and could last for 4–10 h. Over this time, the cycle period of the rhythmic discharge progressively increased. Of all the drug combinations we tested, this one produced the most consistent and regular rhythm (15/16 preparations produced alternating rhythmic activity). The pharmacologically induced rhythm was slower than either spontaneous or dorsal root evoked activity, with an average cycle period of approximately 4 s (Table 2).

Figure 5 illustrates the patterns of left and right L1 ventral root discharge and also shows recordings from other roots.

Although the basic discharge pattern between the contralateral L1/L2 roots was one of alternation, there was some overlap between the firing of contralateral roots (Table 2). This overlap may arise because the L1–L2 roots supply both flexor and extensor muscles (McHanwell and Biscoe 1981). We found that the L3 root discharged with the same pattern as L1 and L2 but that L4/L5 discharge often showed a double-burst pattern that overlapped between the contralateral roots (Fig. 5, C and D). Particularly striking was the discharge of L6 that alternated with the ipsilateral discharge of L1–L3 (Fig. 5, A and B). This finding is similar to the observations of the neonatal rat cord in which rostral lumbar roots (L1–L3) predominately fire during the flexor phase and the L5 discharge occurs during the extensor phase (Iizuka et al. 1997; Kiehn and Kjærulff 1996).

We also investigated the effects of other drug combinations (NMA and 5-HT; dopamine and 5-HT) to establish whether dopamine was essential for rhythmicity and to compare the activity patterns produced by different drug combinations. We were particularly interested in the combination of NMA and 5-HT, which induces rhythmic activity in the rat spinal cord (Cowley and Schmidt 1995, 1997; Kiehn and Kjærulff 1996; Kremer and Lev-Tov 1997). In our initial experiments, application of NMA and 5-HT generated either tonic activity (2/8 preparations) or rhythmic activity that was uncoordinated between the left and right sides (3/8 preparations). In three of

![Figure 5](http://jn.physiology.org/Downloaded from http://jn.physiology.org/)

**FIG. 5.** Bath application of drugs can evoke alternating ventral root discharge. Two examples of rhythmic activity evoked by bath application of 5-HT (10 μM), dopamine (50 μM), and N-methyl-D,L-aspartate (NMA) (5 μM) are illustrated (P2 mice). A: neurograms (0.1–3 kHz) recorded from the L1 and L6 ventral roots following the addition of the drug combination. C: neurograms (0.1–3 kHz) recorded from the L1 and L4 ventral roots following the addition of the drug combination in another animal. B and D: averaged (n = 6 cycles), rectified and smoothed neurograms corresponding to the raw neurograms displayed in A and C.
eight experiments, we were successful in generating alternating rhythmic activity. However, the pattern of activity produced by the drugs was often complex with periods of rhythmic alternation interrupted by more intense tonic discharge.

We found that the episodes of rhythmic activity elicited by NMA and 5-HT differed in several respects from those produced when dopamine was included in the bath (Fig. 6). For example, the cycle period was shorter and the amplitude of the discharge was lower in the absence of dopamine (Table 2). In addition, the alternation between the left and right ventral root discharge tended to be more precise with NMA and 5-HT than with other drug combinations, with less overlap between the discharge of contralateral roots (Fig. 6, Table 2).

When 5-HT and dopamine were added to the bath, a slower rhythm was observed (Table 2) and contralateral ventral root discharges overlapped more than in the presence of NMA and 5-HT alone (Fig. 6).

**RECORDINGS FROM HINDLIMB MUSCLE NERVES SHOW A PATTERN OF FLEXOR-EXTENSOR ALTERNATION DURING DRUG-INDUCED RHYTHMIC ACTIVITY.** We have shown that alternating contralateral ventral root activity can occur spontaneously, be evoked by dorsal root stimulation, or be induced by drugs. In the next set of experiments, we established if the pattern produced following drug application was “locomotor-like” by recording the activity of hindlimb flexor and extensor muscle nerves. To accomplish this, we recorded the discharge of several muscle nerves (tibial, MG, LGS and CP in 9 preparations) for comparison with the previously documented muscle activity during mouse treadmill walking (Fortier et al. 1987).

We were most successful recording muscle nerve activity when the drug concentrations were somewhat higher [75–100 μM dopamine, 15–20 μM 5-HT, 7.5–10 μM NMA (P2–4)] than those used for the ventral root recordings described earlier. Lower concentrations of these drugs either produced tonic activity or produced rhythmic discharge in one, but not both, of the antagonist muscle nerves. No other drug combinations were tried in this set of experiments. In our initial experiments (2/2 preparations), we recorded from the CP and the tibial nerves from both limbs since this recording configuration can demonstrate a pattern of flexor and extensor discharge in the rat (Cowley and Schmidt 1994a). However, we found that the activity pattern of the tibial nerve had a clear double-burst pattern while the CP had a single burst of discharge. In later experiments, we recorded extensor activity from a single ankle extensor muscle nerve (MG) or strict synergists such as the LGS. Recordings from the ankle extensor muscle nerves (LGS or MG) and the ankle flexor CP nerve (3/7 preparations) exhibited an alternating pattern of discharge between the ipsilateral muscle nerves, and also between contralateral CP activity (Fig. 7, A and B). In the remaining four preparations, we recorded either tonic discharge, uncoordinated left/right activity, or unilateral discharge. In one of these four preparations,
we recorded an alternating flexor-extensor pattern in each limb that was uncoordinated between the two sides. Figure 7C summarizes the onset and offset of the contralateral CP and the ipsilateral LGS/MG nerve discharge normalized to the onset of the ipsilateral CP discharge. The LGS/MG discharge occupied a greater portion of the total cycle than the CP discharge (see Fig. 7B). The average cycle period of the drug-induced alternation recorded from muscle nerves was about 3 s (see Table 2).

Collectively, our recordings show that the pattern of drug-induced motoneuron activity is similar to the activity patterns of the same muscles during treadmill locomotion in the mature mouse (Fortier et al. 1987).

CORRELATION OF VENTRAL ROOT AND MUSCLE NERVE ACTIVITY. In the next set of experiments, we compared the discharge patterns of individual muscle nerves with those of the ventral roots to establish the relationship between ventral root discharge and flexor/extensor activity. For this purpose, we recorded LGS and CP nerve activity together with activity from L1 to L2 ventral roots (n = 5 preparations) using the drug combination of NMA, dopamine, and 5-HT. In two of these preparations, we removed the opposite leg to allow easier access to the ventral roots. We found that the flexor CP nerve fired in phase with the ipsilateral L1/L2 ventral root activity and that the extensor LGS nerve fired mainly out of phase with the ipsilateral L1/L2 discharge (Fig. 8C; CP onset relative to ipsilateral L1/L2 onset: 0.07 ± 0.05 SD; CP ipsilateral offset: 0.51 ± 0.06; LGS ipsilateral onset 0.34 ± 0.06; LGS ipsilateral offset: 1.04 ± 0.04, n = 3 preparations). This suggests that L1/L2 discharge is a marker for flexor activity. When the L1 contralateral ventral root was recorded, it fired in phase with the CP activity (Fig. 8B). By inference, this suggests that the ipsilateral L6 ventral root discharge was coactive with the LGS nerve burst.

Dependence of patterned rhythmic activity on intact crossed connections

On occasion (7 preparations), the rhythms on the left and right sides of the cord were desynchronized. In one of these experiments, alternating discharge of flexor and extensor motoneurons was maintained independently on each side.

These experiments suggested that neither rhythmic activity nor unilateral flexor/extensor coordination required synchronized inputs from the opposite side of the cord. To test this hypothesis directly, we examined the capacity of each side of the cord to produce rhythmic activity after a complete middorsal section (6 preparations). We first activated rhythmic activity pharmacologically (5 μM NMA, 10 μM 5-HT, 50 μM dopamine).
dopamine or 75 μM dopamine and 15 μM 5-HT) and then sectioned the cord midsagittally over its entire length. We found that the rhythmic activity persisted on each side of the cord after the lesion in all six preparations (Fig. 9, A and B). The midsagittal section significantly increased the cycle period from a control value of 4.12 ± 0.42 s to a value of 7.02 ± 1.64 s (*P*, 0.001; Fig. 9D). We also found that the rostrocaudal coordination of discharge was preserved after the lesion so that the discharge of the ipsilateral L1 and L6 ventral roots alternated, as it does in the intact cord (Fig. 9, B and C). Collectively, these results suggest that each side of the cord is capable of independently generating rhythmic activity and coordinating the firing of flexor and extensor motoneurons.

In the final set of experiments, we determined if the rhythms induced in each half of the cord required functional NMDA receptors. It has been proposed, in theoretical studies of lamprey rhythmogenesis, that rhythmic activity on each side of the cord could be maintained by NMDA-mediated oscillations (Kotaleski et al. 1999). To test this idea in the neonatal mouse cord, rhythmic activity was first induced in an unlesioned cord with dopamine and 5-HT (n = 6 preparations). We then added the NMDA receptor antagonist AP5 (n = 3) or MK801 (n = 3) and established its effect on the induced rhythmic activity 30 min later. Figure 10A shows that rhythmic activity still occurred in the presence of AP5 (50–100 μM). Blocking NMDA receptors with AP5 did affect the amplitude and the cycle period of the ventral root discharge (Fig. 10, A, B, and D). A similar effect was observed with MK-801 (20–40 μM). As illustrated in Fig. 10A, the phase relations between the contralateral ventral roots were generally maintained during NMDA blockade in six of six preparations. However, during bath application of MK801, the L6, but not the L1/L2, ventral root discharge became either tonic or less coordinated with the rostral segments. In one preparation, the left/right rhythms

![Figure 9](http://jn.physiology.org/)

**FIG. 9.** The alternation between L1/L3 and L6 ventral root discharges is maintained following a complete midsagittal section of the spinal cord. A: rectified and smoothed neurograms obtained during bath application of NMA (7.5 μM), 5-HT (15 μM), and dopamine (75 μM). B: pattern of activation following a complete midsagittal section of the spinal cord. Note that the L6 and L1/L3 discharge continues to alternate after the section. C: phase diagrams summarizing the results from 3 preparations (125–144 cycles per condition). D: the graph shows the increase in cycle period that occurred following midsagittal section of the spinal cord. The results from 2 individual preparations are plotted to indicate the range (min and max) of the data together with the results averaged from all 6 preparations. Error bars indicate the SDs. * Significant difference (*P* < 0.001) between control and lesion. Rhythmic activity was induced by administration of either NMA, 5-HT, and dopamine (3/6) or dopamine and 5-HT (3/6).

![Figure 10](http://jn.physiology.org/)

**FIG. 10.** Rhythmic alternation of ventral root activity can persist after N-methyl-D-aspartate (NMDA) receptor blockade. A, left: the neurograms recorded from the left and right L1 ventral roots (P4 mouse) in the presence of 5-HT (15 μM) and dopamine (75 μM). B: pattern of activation following a complete midsagittal section of the same preparation [rhythm evoked by 5-HT (15 μM) and dopamine (75 μM)] showing the discharge from the left ventral root under control conditions (left), and following addition of AP5 (50–100 μM) to the bath (right). C: quantification of the phase relationships of the discharge patterns for the experiment illustrated in A (14 cycles in each condition). Note that the alternating pattern of discharge remained and that the overlap between the contralateral discharge was reduced. D: bar graph showing that the addition of AP5 to the bath reduced the average length of the cycle period in intact cords and in cords that had a complete mid-sagittal section. Error bars indicate SDs.
became uncoupled during AP5 bath application, but the pattern of alternation between the L2 and L6 ipsilateral ventral roots was retained (data not shown). When the cord was completely sectioned midsagittally over its entire length, the effects of the drugs were similar to those obtained in the unlesioned cord (Fig. 10B). Taken together these results indicate that although NMDA receptors contribute to rhythrogenesis and to the excitatory drive of motoneurons, they are not be essential for the production of rhythmic activity or left/right alternation.

In three experiments, we established the effects of bath application of the AMPA/kainate antagonist 6-cyano-7-nitroquinoxalene-2,3-dione (CNQX). In two of these experiments, we found that rhythmic discharge was abolished following the addition of 20 \( \mu \)M CNQX to the bath. In one experiment where a rhythm persisted after the addition of 20 \( \mu \)M CNQX, the rhythm was blocked when the concentration of the antagonist was increased to 40 \( \mu \)M. This blockade persisted when the concentration of 5-HT was increased in two steps from 20 to 40 \( \mu \)M. Rhythmic activity resumed after washout of the CNQX and reapplication of the drug cocktail in two of three preparations. These results suggest that the network requires functional AMPA/kainate glutamate receptors to generate rhythmic activity consistent with a recent report by Nishimaru et al. (2000).

**Discussion**

In this study we have recorded patterns of ventral root and motor nerve discharge during spontaneous and evoked activity generated by the isolated spinal cord of the neonatal mouse. We have shown that several different patterns of rhythmic motor activity occur spontaneously. In addition, we have demonstrated that alternating left/right rhythmic activity can be induced by stimulation of lumbar and sacral dorsal roots, and that bath-applied drugs can induce a motor pattern characteristic of locomotion. The observation that drug-induced rhythms persist in the hemisected cord and in the presence of NMDA antagonists constrains the types of mechanism that underlie rhythmicity in the neonatal mouse cord.

**Spontaneous activity expressed by the neonatal mouse cord**

Spontaneous motor output recorded from the isolated lumbosacral cord was complex. It comprised highly variable ventral root depolarizations that were sometimes accompanied by a brief episode of rhythmic bursting (Fig. 1; see also supplementary data on-line, http://jn.physiology.org/cgi/content/full/84/6/2821/DC1). During these episodes, several patterns of discharge could occur including: left/right alternation (Bonnot et al. 1998a), unilateral bursting and subthreshold oscillations that were synchronized over many segments (Fig. 2). In this investigation, most of the animals were aged P2–4 so that we did not establish if any of these patterns changed with age.

We do not know the significance of these various patterns of activity, but an exciting possibility is that they represent the transient expression of different motor behaviors (scratching, locomotion etc). Another possibility is that the various patterns represent the expression of components of particular behaviors rather than complete behaviors. Consistent with this idea, we found that various aspects of the motor behavior (left/right alternation, flexor/extensor alternation) could occur independently of each other. For example, rhythmic ventral root discharges do not require the presence of left/right alternation. Similarly, flexor/extensor alternation does not require left/right alternation because it can occur independently on both sides of the cord or in the hemisected cord. Finally we have evidence that rhythmic activity of some flexor and extensor motoneurons can be uncoupled because we recorded instances of isolated flexor or extensor motoneuron discharge.

Collectively, these results suggest a surprising level of independence between the various components of the motor output that may reflect the immaturity of the underlying networks. Apparently at this stage of development, the various synergies characteristic of adult motor patterns are more fluid and independent than in the mature state. It will be important in future work to document how, and by what mechanisms, the various patterns become constrained to give rise to the mature behaviors. Consistent with the hypothesis of circuit immaturity, we observed one pattern that is generally characteristic of fetal or embryonic networks. In this pattern, fast subthreshold slow potential oscillations were recorded from the ventral roots, and these were synchronized (cycle by cycle) throughout the lumbar and sacral cord. These oscillations resemble the early spontaneous activity of both the rat and chick spinal cords (O'Donovan and Landmesser 1987) and may represent the persistence of a fetal activity pattern as spinal networks are assembling the more coordinated patterns characteristic of mature motor behavior.

**Drug-induced locomotor-like activity**

In the presence of dopamine, 5-HT and NMA, rhythmic activity was characterized by an alternation between contralateral ventral roots (L1–L3, L6) and between the flexor common peroneal and the extensor lateral gastrocnemius/soleus muscle nerves. This pattern of discharge resembles that recorded electromyographically from hindlimb flexor and extensor muscles in walking mice (Fortier et al. 1987; Hernandez et al. 1991). Hernandez et al. (1991) recorded from the gastrocnemius and tibialis anterior muscles of freely moving neonatal and adult mice and showed that their activity alternated during locomotion, although the phase relations between the antagonist muscles were more labile in the neonate than in the adult. Similarly, Fortier et al. (1987) recorded the electromyographic activity of hindlimb flexor (tibialis anterior) and extensor (medial gastrocnemius) muscles during treadmill locomotion in the adult mouse and found an alternating pattern of activity similar to the pattern we recorded between the common peroneal and lateral gastrocnemius/soleus muscle nerves.

It is unclear why drug activation resulted in the expression of locomotor-like discharges rather than any of the other spontaneous motor patterns that we observed. One possibility is that locomotor networks have a high threshold for activation (requiring drugs) and once active, inhibit other circuits. Consistent with this idea, we found that spontaneous alternating activity tended to be associated with the largest spontaneous depolarizations, suggesting that the circuitry responsible for this type of behavior required more intense activation than the networks underlying other patterns of discharge. In addition, we did not observe mixed rhythms in which one pattern was co-expressed with another, suggesting each pattern was expressed separately.
Our observations suggest that the networks activated pharmacologically produce a coordinated motor output that is similar to that recorded in vivo during adult locomotion. However, this does not imply that the underlying networks are similar, and as we have discussed, there are several reasons for believing that the neonatal networks are still in a process of maturation.

**Mechanisms of rhythmic activity in the neonatal mouse cord**

Reciprocal inhibitory connections between left and right rhythmogenic centers have been proposed as an important component underlying rhythmogenesis in the lamprey and tadpole spinal cords (Grillner et al. 1998; Sillar et al. 1998). Such connections between flexor and extensor centers have also been implicated in rhythmogenesis underlying scratching in the turtle (Mortin and Stein 1990). Two observations from the present work suggest that this mechanism is not essential for rhythmogenesis in the neonatal mouse cord. First, we found that rhythmic discharge could sometimes occur independently on the two sides of the cord. Second, rhythmic activity, with appropriate L1/L6 alternation persisted in the hemisected cord. These findings are in agreement with earlier reports in the mouse (Tao and Droge 1992) showing that midsagittal section of the cord did not substantially alter the incidence of spontaneous rhythmic electromyographic activity in isolated spinal cord/hindlimb preparations of neonatal mice. Similar findings have been made in the neonatal rat in which one side of the cord is selectively activated (Kjaerulf and Kiehn 1997) or hemisected (Kremer and Lev-Tov 1997). Of course the ability of each side of the cord to generate rhythmic activity independently does not exclude the involvement of reciprocal left-right inhibition in rhythmogenesis, but it is clearly not essential. One possible function for left/right inhibition could be to synchronize ongoing left/right rhythms in antiphase rather than being causally involved in rhythmogenesis.

In other species, NMDA receptors have been postulated to play an important role in rhythmogenesis (Alford and Grillner 1990; Beato et al. 1997; Grillner et al. 1998; MacLean et al. 1997; Reith and Sillar 1998; Schmidt et al. 1998). In the lamprey spinal cord, this mechanism has been proposed to play a role in rhythm generation by the hemisected cord in which the reciprocal inhibitory interactions with the other side have been abolished (Kotaleski et al. 1999). In the present work, application of the NMDA antagonist AP5 or MK801 did not abolish rhythmic activity in either the intact (unlesioned) or the hemisected cord (Fig. 10), although the drugs did influence both the amplitude and the frequency of the remaining activity. While these observations implicate the participation of NMDA receptors in some aspect of the motor output, they also show that these receptors are not required for bursting on one side of the cord. In contrast, we found that administration of CNQX blocked all ventral root output in three of three preparations, suggesting that the underlying networks probably employ AMPA/kainate receptors for their excitatory connections. These results are consistent with a recently published report by Nishimaru et al. (2000) showing that rhythmic activity induced by 5-HT was abolished by bath application of kynurenic acid, a NMDA and AMPA/kainate receptor blocker, but not by application of the NMDA receptor antagonist AP5.

We found that rhythmic activity could be generated by isolated segments of both the lumbar (unpublished observations) and sacral spinal cords (Fig. 3B), confirming and extending earlier reports (Bonnot and Morin 1998). These findings indicate that the capacity for rhythmogenesis is distributed along the lumbo-sacral spinal cord in the neonatal mouse, as it is in other species (Grillner et al. 1998; Ho and O’Donovan 1993; Kjaerulf and Kiehn 1996; Lev-Tov and Kremer 1999; Lev-Tov et al. 2000; Mortin and Stein 1989; Roberts et al. 1998; Stein et al. 1998). We have presented evidence that the lumbar rhythm evoked by drugs resembles the motor synergies characteristic of locomotion, but the function of rhythmic circuitry in the sacral cord is less clear. Recently in the neonatal rat spinal cord, it has been shown that afferent stimulation can induce rhythmic discharges in the isolated sacral cord and that these appear to control tail movements (Lev-Tov et al. 2000). It is possible, therefore, that the sacral cord rhythmonic circuitry subserves a similar function in the neonatal mouse.

**Comparison of motor patterns generated by the neonatal mouse and rat spinal cords**

In the neonatal rat spinal cord, locomotor-like activity can be induced either by NMDA and 5-HT alone or by both drugs in combination (Cazalets et al. 1992, 1995; Cowley and Schmidt 1994b; Kudo and Yamada 1987). We found that 5-HT and NMA could sometimes produce periods of rhythmic alternation, but usually the induced rhythm was not well synchronized between the two sides of the cord. Robust locomotor-like activity required the presence of dopamine in the bath, although the ensuing rhythms were substantially slower than those induced by 5-HT and NMA (Fig. 6). Bath application of 5-HT and dopamine alone could also activate an alternating rhythm, but again it was slower than that produced when all three drugs were combined. Similar findings have been made in the isolated rat cord in which dopamine alone can induce rhythmic activity that is much slower than that induced by 5-HT alone (Kiehn and Kjaerulf 1996). In the rat, electromyographic recordings from many hindlimb muscles have shown that different rhythmic patterns are induced by 5-HT and dopamine. Some muscles (e.g., the functional hip extensors and knee flexors, biceps femoris, and semitendinosus) exhibited a flexor-like discharge pattern in the presence of dopamine but an extensor-like pattern in the presence of 5-HT. The pattern evoked by 5-HT in the rat was most similar to swimming while that evoked by dopamine was closer to locomotion (Kiehn and Kjaerulf 1996). Our work in the mouse has not been detailed enough to establish if the various drug combinations induce similar patterns in the mouse, but we did notice a greater degree of overlap between the left and right ventral root discharge when dopamine was included in the bath. Double-burst patterns were not observed in a recent study in the mouse in which 5-HT alone was used to activate the rhythm (Nishimaru et al. 2000). A definitive interpretation of the various drug-induced patterns of activity must await an understanding of the mechanisms underlying the induced rhythmic activity.

In conclusion, our findings have revealed an unexpected complexity in the motor output generated by the neonatal mouse spinal cord in vitro. In future experiments, it will be necessary to establish whether or not these patterns correspond to recognizable behaviors and to begin to define the circuitry...
underlying the activity. The ability to study rhythmically active networks under several different conditions offers great promise for future studies of mammalian locomotor circuitry.

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