Ontogeny of Rhythmic Motor Patterns Generated in the Embryonic Rat Spinal Cord

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Ren, Jun, and John J. Greer. Ontogeny of rhythmic motor patterns generated in the embryonic rat spinal cord. J Neurophysiol 89: 1187–1195, 2003; 10.1152/jn.00539.2002. Patterned spontaneous activity is generated in developing neuronal circuits throughout the CNS including the spinal cord. This activity is thought to be important for activity-dependent neuronal growth, synapse formation, and the establishment of neuronal networks. In this study, we examine the spatiotemporal distribution of motor patterns generated by rat spinal cord and medullary-spinal cord systems from the time of initial axon outgrowth through to the inception of organized respiratory and locomotor rhythmogenesis during late gestation. This includes an analysis of the neuropharmacological control of spontaneous rhythms generated within the spinal cord at different developmental stages. In vitro spinal cord and medullary-spinal cord preparations isolated from rats at embryonic ages (E13.5–E21.5) were studied. We found age-dependent changes in the spatiotemporal pattern, neurotransmitter control, and propensity for the generation of spontaneous rhythmic motor discharge during the prenatal period. The developmental profile of the neuropharmacological control of rhythmic bursting can be divided into three periods. At E13.5–E15.5, the network comprising cholinergic and glycnergic synaptic interconnections is capable of generating rhythmic activity, while GABAergic synapses play a role in supporting the spontaneous activity. At late stages (E18.5–E21.5), glutamate drive acting via non-N-methyl-D-aspartate (non-NMDA) receptors is primarily responsible for the rhythmic activity. During the middle stage (E16.5–E17.5), the spontaneous activity results from the combination of synaptic drive acting via non-NMDA glutamatergic, nicotinic acetylcholine, glycine, and GABA A receptors. The modulatory actions of chloride-mediated conductances shifts from predominantly excitatory to inhibitory late in gestation.

METHODS

In vitro prenatal rat preparations

Embryos (E13.5–E21.5; n = 117) were delivered from timed-pregnant rats anesthetized with halothane (1.2–1.5% delivered in 95% O 2 -5% CO 2 ) and maintained at 37°C by radiant heat following procedures approved by the Animal Welfare Committee at the University of Alberta. The timing of pregnancies of dams was determined from the appearance of sperm plugs in the breeding cages. The ages of fetuses were confirmed by comparison of their crown-rump length measurements with those published by Angulo and González (1932). Immediately on delivery, the neuroaxis was isolated from embryos as previously described (Greer et al. 1992). The spinal cord was dissected to include segments extending from the first cervical (C 1 ) to the fourth sacral (S 4 ) ventral roots. In other preparations, the medulla was left attached. The preparations were continuously perfused at 27 ± 1°C (perfusion rate 5 ml/min, volume of the chamber 1.5 ml) with mock cerebrospinal fluid (CSF) that contained (mM) 128 NaCl, 3.0 or 5.0 KCl, 1.5 CaCl 2 , 1.0 MgSO 4 , 24 NaHCO 3 , 0.5 NaH 2 PO 4 , and 30 D-glucose equilibrated with 95% O 2 -5% CO 2 (pH = 7.4).

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TABLE 1. Interval and duration of spontaneous bursts recorded before and after spinal cord transection at T₁ and T₁₃

<table>
<thead>
<tr>
<th>Age</th>
<th>n</th>
<th>Cervical Cord</th>
<th>Thoracic Cord</th>
<th>Lumbar Cord</th>
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<td></td>
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<td>Intact</td>
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<tr>
<th>Interval, min</th>
<th>1.9 ± 0.2</th>
<th>2.9 ± 0.6*</th>
<th>1.9 ± 0.2</th>
<th>2.1 ± 0.5</th>
<th>1.9 ± 0.2</th>
<th>5.2 ± 1.7*</th>
</tr>
</thead>
<tbody>
<tr>
<td>E14.5</td>
<td>2.8 ± 0.5</td>
<td>5.9 ± 1.8*</td>
<td>2.8 ± 0.4</td>
<td>4.3 ± 1.0*</td>
<td>2.8 ± 0.5</td>
<td>4.5 ± 1.0*</td>
</tr>
<tr>
<td>E15.5</td>
<td>3.2 ± 0.9</td>
<td>20.5 ± 8.4*</td>
<td>2.7 ± 0.6</td>
<td>14.3 ± 6.5*</td>
<td>2.5 ± 0.5</td>
<td>3.0 ± 1.1</td>
</tr>
<tr>
<td>E16.5</td>
<td>12.7 ± 4.6</td>
<td>&gt;60*</td>
<td>4.5 ± 1.3</td>
<td>&gt;60*</td>
<td>2.8 ± 1.3</td>
<td>4.9 ± 2.3</td>
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<tr>
<td>E17.5</td>
<td></td>
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<tr>
<td>Burst duration, s</td>
<td>2.7 ± 0.3</td>
<td>2.4 ± 0.5</td>
<td>3.5 ± 0.4</td>
<td>3.2 ± 0.6</td>
<td>2.1 ± 0.4</td>
<td>2.0 ± 0.5</td>
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<tr>
<td></td>
<td>6.1 ± 0.6</td>
<td>6.4 ± 0.8</td>
<td>6.3 ± 0.6</td>
<td>6.8 ± 0.7</td>
<td>5.2 ± 0.7</td>
<td>5.0 ± 0.6</td>
</tr>
<tr>
<td>E16.5</td>
<td>13.8 ± 3.2</td>
<td>19.5 ± 2.1*</td>
<td>13.5 ± 2.4</td>
<td>16.4 ± 3.3</td>
<td>10.4 ± 2.4</td>
<td>9.1 ± 0.5</td>
</tr>
<tr>
<td>E17.5</td>
<td>21.6 ± 6.2</td>
<td>N/A</td>
<td>15.3 ± 5.8</td>
<td>N/A</td>
<td>11.6 ± 3.5</td>
<td>9.3 ± 3.6</td>
</tr>
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</table>

Values are means ± SD; * P < 0.05 compared with intact.
The interval between spontaneous bursts was significantly \( (P < 0.05) \) prolonged in the cervical and lumbar segments by transection at E14.5, while the interval between bursts in the thoracic segment was not significantly affected and remained the most robust. At E15.5, transections prolonged the burst interval in each spinal cord segment. After transections at E16.5, the interval between bursts in cervical and thoracic segments was significantly increased \( (P < 0.05) \), while the interval between lumbar cord bursts was similar to control values and was most robust.

### Age-dependent changes in the neurochemical control of patterned rhythmic activity

To determine the role of various neurotransmitters in the generation and modulation of patterned rhythmic discharge, we applied neurotransmitter receptor agonists, antagonists, and uptake inhibitors to the bathing medium at different developmental ages. Specifically, we assayed for the roles of acetylcholine, glycine, GABA, and glutamate by recording rectified integrated motor discharge. It should be noted that these data did not provide information on interneuronal or subthreshold rhythmic motor activity that may have persisted in the presence of the various pharmacological manipulations.

#### ACETYLCHOLINE

As shown in Fig. 4 A and B, rhythmic motor patterns were inhibited in the presence of the nicotinic acetylcholine receptor antagonist d-tubocurarine (dTC; 20 \( \mu \)M) at ages E13.5–E17.5. The rhythmic activity in E14.5–E15.5 preparations was also blocked by nicotinic receptor antagonists mecamylamine (15 \( \mu \)M; \( n = 2 \)) and dihydro-beta-erythroidine (10 \( \mu \)M; \( n = 2 \)). The activity was inhibited for the duration of the recording session (30–60 min). Beyond E17.5, dTC had little or no effect on rhythmic motor discharge. The rhythm was not affected by the muscarinic receptor antagonist atropine (1–10 \( \mu \)M) at any ages. To further examine the actions of acetylcholine, we administered the cholinesterase inhibitor eserine (1 \( \mu \)M) to the bathing medium (Fig. 4C). Increasing the endogenously released levels of acetylcholine with a 5-min application of eserine resulted in an increase in the duration of rhythmic bursts by 380 \( \% \) (\( n = 5 \)) and 160 \( \% \) (\( n = 6 \)) at ages E14.5 and E16.5, respectively. There was also a transient increase in the frequency of rhythmic bursting that was typically followed by a rebound depression.

#### GLYCINE

Figure 5 illustrates the age-dependent changes in the role of glycinergic transmission in the generation of spontaneous rhythmic motor discharge. Blocking glycine receptors with strychnine (STR; 25 \( \mu \)M) abolished the rhythmic discharge at ages E13.5–E17.5. The activity was inhibited for the duration of the recording session (30–60 min). In contrast, blocking glycinergic receptors post-E18.5 led to an increase in the frequency of rhythmic discharge.

#### GABA

The age-dependent changes resulting from the blocking of GABA \(_A\) receptors with bicuculline (BIC; 50 \( \mu \)M) are illustrated in Fig. 6. At ages E13.5–E15.5, bicuculline caused a 53 \( \% \) (\( n = 13 \)) reduction in the frequency of rhythmic bursts. At E16.5, the same dose of bicuculline reduced the frequency to 12 \( \% \) (\( n = 5 \)) of control. The rhythmic activity was completely inhibited in four of five preparations at E17.5. Post-E17.5, blocking GABA \(_A\) receptors either had no effect or increased the frequency of rhythmic bursts (Fig. 6, C and D).

#### AGE-DEPENDENT CHANGES IN CHLORIDE-MEDIATED CONDUCTANCES

Pre-E17.5, activation of GABA \(_A\) receptors (Fig. 7, A and C) with muscimol (0.3 \( \mu \)M) resulted in an increase in the fre-
frequency of rhythmic discharge. In contrast, at late stages of embryonic development (more than E19), activation of chloride-mediated conductances decreased the frequency of rhythmic motor discharge (Fig. 7, B and C). Increasing the endogenous levels of glycine with sarcosine (1 mM; \(n = 11005\)) at E14.5 resulted in an increase of the frequency of rhythmic discharge by \(181 \pm 52\%\) (Fig. 7D).

**GLUTAMATE.** The spontaneous rhythmic discharge was not altered at ages E13.5–E15.5 in the presence of the non-NMDA receptor antagonist CNQX (6 \(\mu M\); Fig. 8). At E16.5, CNQX transiently abolished the rhythm in cervical motor populations in all seven preparations studied. However, the rhythm reappeared within 10–20 min despite the continued exposure to CNQX. The amplitude of activity at E16.5 was decreased to \(65 \pm 13\%\) (\(n = 7\)) of control on thoracic roots, but the frequency of rhythms was unaltered on thoracic and lumbar roots. At E17.5, CNQX abolished the rhythm on cervical, thoracic and lumbar roots. However, the spontaneous activity re-emerged on lumbar roots after approximately 15–20 min despite continued exposure to CNQX; activity remained absent on thoracic and cervical roots during the recording session (approximately 30 min). The rhythmic activity in E18.5–E21.5 preparations bathed in Krebs solution containing 5 mM potassium was blocked by CNQX but not by antagonists to acetylcholine, glycine, or GABA\(_A\) receptors. In fact, as reported in the preceding text, blocking of chloride-mediated conductances caused an increase in the frequency of rhythmic bursts. Blocking of NMDA receptors with the antagonist APV (30–100 \(\mu M\); \(n = 8\)) and MK-801 (100 \(\mu M\); \(n = 2\)) did not alter the rhythmic discharge at any age studied.

**COMBINATORIAL ACTIONS OF NEUROTRANSMITTERS.** It was clear from the data presented in the preceding text that multiple 

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**FIG. 4.** Contribution of nicotinic acetylcholine receptor-activation to the generation of spontaneous rhythmic bursting. A: application of the nicotinic receptor antagonist \(\alpha\)-tubocurarine (dTC; 20 \(\mu M\)) resulted in the suppression of rhythmic bursting at ages E14.5 and E17.5 but had no significant effect at E19.5 (bathed in 5 mM extracellular potassium). B: population data showing the percent changes in the frequency of bursting relative to control in response to dTC (20 \(\mu M\)) at different prenatal ages. Each data point is from 4–6 preparations. C: the duration and frequency of rhythmic bursting was increased after the addition of the cholinesterase inhibitor eserine (1 \(\mu M\)) to an E14.5 spinal cord preparation.

**FIG. 5.** Contribution of glycinergic transmission to the generation of spontaneous rhythmic bursting. Application of the glycine receptor antagonist strychnine (STR, 25 \(\mu M\)) abolished the rhythmic bursting at ages E15.5 (A) and E17.5 (B). At E21.5 (C), the same concentration of strychnine caused an increase in the frequency of rhythmic bursting (bathed in 5 mM extracellular potassium). D: population data showing the percent changes in the frequency of bursting relative to control in response to strychnine (25 \(\mu M\)) at different prenatal ages. Each data point is from 5 to 7 preparations.
neurotransmitters were contributing to the generation of rhythms prior to E18.5. We investigated this further by performing a series of recordings from E16.5 preparations in the presence of multiple receptor antagonists in combination with agents that increased the endogenous levels of neurotransmitter (Fig. 9). In Fig. 9A, we demonstrate the interactions of glycine and non-NMDA receptor mediated effects. The addition of 10 μM strychnine (n = 6) to the perfusate caused a decrease (40 ± 11%; n = 6) of frequency of rhythmic motor discharge. Application of CNQX (6 μM) caused a marked decrease in the frequency (86 ± 6%; n = 6) of rhythmic activity on cervical motor pools with little effect on lumbar populations (decrease of 9 ± 10%). However, the combination of 10 μM strychnine and 6 μM CNQX abolished the rhythmic discharge in all spinal segments in all six preparations tested. The inhibition of rhythmic activity by higher concentrations of strychnine (25 μM) was countered by elevating the endogenous levels of acetylcholine with the cholinesterase inhibitor eserine (2 μM) in four of the six preparations tested (Fig. 9, B and D). The rhythm was reduced in frequency, but persisted, in the presence of strychnine (25 μM), CNQX (6 μM), and bicuculline (50 μM) in all four preparations tested. The rhythm was finally abolished with subsequent application of dTC (40 μM) in three of the four preparations tested. The combinatorial actions of acetylcholine and glycine are further emphasized in Fig. 9, C and E. The blocking of the rhythm by dTC (20 μM) was reversed by elevating endogenous glycine levels with sarcosine (2 mM). The rhythm was slowed by d-tubocurarine (20 μM), CNQX (6 μM), and bicuculline (50 μM) and finally abolished by strychnine (40 μM) in four of five preparations tested.

Interaction of rhythmic patterns generated by brain stem-spinal cord preparations during late gestation

In 19 of 25 brain stem-spinal cords isolated from E13.5 to E16.5 rats, the rhythm motor pattern recorded from spinal ventral roots was also present on hypoglossal (XII) roots (Fig. 10). As described previously (Greer et al., 1992), brain stem-
spinal cord preparations start generating respiratory rhythms on XII and cervical roots at E17. We were interested in examining the interaction of the widespread spinally generated rhythms with the more spatially restricted and regulated respiratory rhythm. Figure 10 shows the spontaneous respiratory rhythm recorded from the hypoglossal (XII) rootlet and the longer-duration bursts on lumbar ventral roots in an E18.5 preparation. The respiratory rhythm was occluded in the presence of the spinal rhythm in all four preparations examined.

**DISCUSSION**

The embryonic rat spinal cord generates robust spontaneous rhythmic patterns that spread throughout the full extent of the spinal cord and into the medulla. The spatiotemporal organization of the pattern undergoes significant transformations during the period spanning E13.5–E18.5. The propensity for the generation of embryonic rhythms declines markedly during late gestation when the more spatially restricted and organized respiratory and locomotor patterns emerge. Multiple neurotransmitters act in concert to provide the excitatory drive necessary to generate rhythms prior to E18.5. Post-E18.5, the rhythms are generated primarily via glutamatergic transmission.

**Spatiotemporal development of spontaneous rhythmic bursts**

The data demonstrating that rhythmic patterns are generated as early as E13.5 in rostral spinal segments and at E14.5 in lumbar segments is consistent with results from previous work (Greer et al. 1992; Nakayama et al. 1999). However, past studies were rather incomplete and did not include recordings from motor pools along the full rostrocaudal extent of the brain stem and spinal cord. Thus the fact that the thoracic segments were a focus for the inception of spinal rhythmogenesis at early embryonic stages and that spontaneous rhythmic patterns spread into medullary motoneuron populations was not realized. The transection experiments demonstrated that cervical, thoracic, and lumbar segments were all capable of generating spontaneous rhythmic patterns. However, the thoracic and lumbar segment circuitry produced the most robust rhythms during early and late gestational ages, respectively.

At E13.5, when spontaneous rhythmic activity was first recorded, motor axons are migrating to and initiating contact with primordial muscle (Allan and Greer 1997). Spontaneous rhythm bursting commences at a similar stage of chick spinal cord development (Milner and Landmesser 1999). The activity in the prenatal rat preparations persists until E18.5, which encompasses periods of intramuscular nerve branching, establishment of functional neuromuscular junctions, and myotube formation (Allan and Greer 1997; Bennett and Pettigrew 1974; Laskowski and Owens 1994; Noakes et al. 1983; Ross et al. 1987). Motoneurons also undergo some key developmental events during this period, including marked changes in the expression of voltage-dependent channels and neurotransmitter receptor subunits and a significant reduction in numbers via the process of naturally occurring cell death (Harris and McCaig 1984; Martin-Carballo and Greer 1999, 2000, 2001; Martin-Carballo et al. 2000; Ross et al. 1987; Xie and Ziskind-Conhaim 1995; Ziskind-Conhaim 1988). Beyond E17.5–E18.5, the spontaneous rhythms were seldom observed in preparations bathed in solutions containing physiological levels of extracellular potassium (3 mM). The timing of the decline of the widespread rhythms correlates with the inception of respiratory and alternating locomotor rhythm discharge (Greer et al. 1992; Kobayashi et al. 2001; Ozaki et al. 1996). As illustrated in Fig. 10, the occurrence of the widespread motor discharge characteristic of early embryonic ages interferes with and occludes the respiratory pattern. This would be functionally inappropriate, resulting in the perturbation of fetal breathing movements that are critical for proper development of lung, motoneuron and muscle properties (Greer et al. 1999; Jansen and Chernick 1991; Kitterman 1996).

The underlying mechanisms responsible for the decline in the spontaneous rhythm pattern during late gestation are likely multifactorial. The spontaneous rhythmic motor patterns arise from the combination of neurochemical synaptic events.
and electrical coupling (Milner and Landmesser 1999; Tresco and Kiehn 2000). There is a major transition in the neurotranschemical component that occurs by E18.5 that includes a restriction to a purely glutamatergic-mediated rhythmenogenesis. There may be also a reorganization and/or pruning of synaptic connections along the rostrocaudal extent of the neuraxis. Electrical coupling persists during late gestation and into the newborn period (Chang et al. 1999; Fulton et al. 1980; Martin-Carballo and Greer 1999). However, the coupling during the perinatal period is largely restricted to motoneurons innervating the same skeletal muscle (Walton and Navarrete 1991). Data from recent studies by Personius et al. (2002) demonstrate that at earlier embryonic ages, gap junctions among spinal neurons are much more widespread and thus likely to facilitate the spread of spontaneous activity.

**Development of the neuropharmacological control of rhythmic bursts**

The developmental profile of the neuropharmacological control of rhythmic bursting can be divided into three periods. At E13.5–E15.5, the spinal networks comprising cholinergic and glycinergic synaptic interconnections are capable of generating rhythmic activity, while GABAergic synapses play a role in supporting the spontaneous motor activity. It appears that each of the neurotransmitters contributes partial excitatory drive necessary for rhythm generation. Removal of anyone of the three can result in an inhibition or failure to achieve threshold for inducing rhythmic motor drive. These results are consistent with those of Milner and Landmesser, who found that cholinergic and GABAergic transmission contributed to the genesis of spontaneous rhythmic bursting during early stages of chick spinal cord development. The potential role of glycine receptors was not reported in that study.

At late stages (E18.5–E21.5), glutamatergic drive acting via non-NMDA receptors is primarily responsible for the rhythmic activity. This is consistent with what has been reported in past studies of chick (Barry and O’Donovan 1987) and rat spinal cord rhythmic activity (Nakayama et al. 1999). Further, a similar developmental transition from cholinergic to glutamatergic-mediated rhythm has been reported for the spontaneous bursting in the retina (Wong et al. 1998).

During the middle stage (E16.5–E17.5), the spontaneous activity involves synaptic drive acting via non-NMDA glutamatergic, nicotinic acetylcholine, glycine, and GABA receptors. All of these transmitter systems provide a component of excitation necessary to achieve rhythmicity. Blocking of any one of the receptor species can result in the loss of rhythm that can be subsequently re-established by increasing the endogenous levels of one of the other neurotransmitters. In the developer
opining chick spinal cord, blockade of rhythmic activity via antagonists to one class of receptors can also be transient, with the rhythm re-emerging under the control of a different neurotransmitter (Chub and O’Donovan 1998; Milner and Landmesser 1999).

The actions of chloride-mediated conductances changed with embryonic age. The net effect of activation of GABA$_A$ and glycine receptors depends on the distribution of chloride ions across the membrane and the degree of shunting of other receptor-mediated currents. At the early and mid stages of embryonic development (i.e., preE18.5), activation of chloride-mediated conductances caused an increase in the frequency of spontaneous rhythmic bursting. At later stages, the rhythms were suppressed in the presence of increasing endogenous levels of glycine or the presence of agonists to GABA$_A$ or glycine receptors.

Glycine, GABA, and glutamate are expressed in neurons within the developing spinal cord (Ma et al. 1992; Schaffner et al. 1993; Wu et al. 1992; Ziskind-Conhaim 1990). The identity of the specific neurons within those populations that are responsible for the generation of embryonic spinal rhythms is not known. The source of cholinergic synaptic inputs involved in rhythmogenesis at early stages is particularly puzzling. There have been not any reports of a network of cholinergic cells that are expressed transiently within the spinal cord that could account for the data from this and the chick studies. Milner and Landmesser (1999) hypothesize that the source of cholinergic input may arise from synaptic inputs onto interneurons and motoneurons from arborizations of motoneuronal axons (Perrins and Roberts 1995), paracrine-like release of acetylcholine from motoneurons, or cholinergic release from partition cells located near the central canal (Phelps et al. 1991).

In summary, prior to the inception of organized respiratory and locomotor rhythms in the spinomedullary axis, there are robust rhythmic motor discharge patterns generated within the spinal cord of the embryonic rat. The frequency and spinal loci of initiation of these spontaneously generated rhythms undergoes characteristic changes during the final week of gestation. There are also marked changes in the neurotransmitters responsible for the genesis and modulation of rhythmic bursting. The neuronal firing, neurotransmitter release and signaling via gap junctions associated with spontaneous rhythmic motor patterns will result in ionic flux, release of neurotrophic signals and the modification of the intracellular milieu throughout the neuraxis. Collectively, these could contribute to the regulation of phenotypic changes of cellular and network properties during the formative embryonic period.

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REFERENCES


