Rhythmic Neuronal Discharge in the Medulla and Spinal Cord of Fetal Rats in the Absence of Synaptic Transmission

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Rhythmic neuronal discharge in the medulla and spinal cord of fetal rats in the absence of synaptic transmission. J Neurophysiol 95: 527–534, 2006. First published September 7, 2005; doi:10.1152/jn.00735.2005. Spontaneous rhythmic neuronal activity is generated in the developing vertebrate nervous system. The patterned activity spreads diffusely throughout the fetal neuraxis. Here we demonstrate the ability of the fetal rat spinal cord and medulla to generate and transmit robust rhythmic patterns in the absence of synaptic activity. Regular rhythmic discharges were produced by fetal tissue bathed in low or zero [Ca\(^{2+}\)] solution. The activity persisted in the presence of antagonists to neurotransmitter receptors that are known to mediate synaptic-mediated events associated with fetal rhythms. A combination of ventral root recordings and optical imaging using voltage-sensitive dyes demonstrated the extensive spread of rhythmic discharge in spinal cord and medullary neuronal populations of in vitro preparations. Whole cell recordings from medullary slices were performed to examine the ionic conductances and revealed the importance of persistent sodium conductances for generation of rhythmic activity in hypoglossal (XII) motoneurons. Rhythmic bursting in XII motoneurons persisted in the presence of gap junction blockers, although the amplitude of synchronized motor discharge recorded from nerve roots was diminished. We propose that nonsynaptically mediated conductances, potentially by extracellular ionic flux and/or ephaptic and electrotonic interactions mechanisms, act in concert with neurochemical transmission and gap junctions to promote the diffuse spread of rhythmic motor patterns in the developing nervous system.

INTRODUCTION

Episodes of spontaneous rhythmic activity are widespread in the developing vertebrate nervous system (Hanson and Landmesser 2003; Katz and Shatz 1996; Milner and Landmesser 1999; Nakayama et al. 1999; O’Donovan 1999; Ren et al. 2003; Yvert et al. 2004). The rhythms are a distinct patterned activity that are proposed to serve a critical role in a variety of developmental processes including neurite path finding, synaptogenesis, establishment of neuronal networks, axonal pruning, maturation of neuronal electrophysiological properties, and the release of neurotrophic factors (Ba et al. 2005; Dahm and Landmesser 1991; Hanson and Landmesser 2004; Spitzer and Ribera 1998; Xie and Ziskind-Conhaim 1995). The rhythmic activity commences from early stages of development when axons are path finding to their targets through to the late stages of gestation with the inception of more specialized and spatially restricted neuronal activity associated with respiratory and locomotor patterns. Redundant neurotransmitter systems

mediate synaptic drive associated with the generation of motor patterns in the developing neuraxis (Chub and O’Donovan 1998; Milner and Landmesser 1999; Nishimaru et al. 1996; Ren and Greer 2003). Further, motoneurons are connected by gap junctions prenatally that facilitate the synchronization and spread of motor discharge (Bittman 2004; Tresch and Kiehn 2000). However, a striking feature of the fetal rhythmic oscillations that deserves consideration is the extent to which the activity radiates throughout the developing neuraxis. The synchronized activity spreads along the full extent of the spinal cord and into ventral and dorsal medullary nuclei (Greer et al. 1992; Yverts et al. 2004). There are nonsynaptic mechanisms for propagating neuronal activity that could conceivably play an important role in the spread of network rhythmic discharge. These include extracellular potassium waves associated with neuronal bursting and ephaptic or electrotonic interactions involving large extracellular fields that alter the excitability of neighboring neurons (Dudek et al. 1998; Jeffreys 1995). Here we demonstrate the ability of the fetal rat spinal cord and medulla to generate and widely transmit robust neuronal activity in the absence of synaptic activity, lending support for such proposed mechanisms.

METHODS

In vitro prenatal rat preparations

Fetuses (n = 103) were delivered from timed-pregnant Sprague–Dawley (n = 92) and Wistar (n = 11) rats anesthetized with halothane (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. Immediately on delivery, the neuraxis was isolated from fetuses as previously described (Greer et al. 1992). Spinal cord–brain stem preparations: The spinal cord and brain stem were dissected to include segments extending from the medulla to the fourth sacral (S4) ventral roots or, in preparations without the medulla, from the first cervical (C1) to sacral levels. In some preparations transverse slices containing one to two lumbar segments were prepared. Medullary slice preparations: The brain–spinal cords isolated from fetal rats as described above were pinned down, ventral surface upward, on a paraffin-coated block. The block was mounted in the vise of a vibratome bath (Leica VT1000S) and a single transverse slice was cut (approximately 500 \(\mu\)m thick), transferred to a recording chamber, and pinned down onto a Sylgard elastomer. Bathing solutions: All preparations were continuously perfused at 28 ± 1°C (perfusion rate 5 ml/min, volume of the chamber 1.5 ml) with

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modified Kreb’s solution that contained (in mM): 128 NaCl, 5.0 (brain stem–spinal cord preparations) or 9.0 (spinal cord or medullary slice preparations) KCl, 1.5 CaCl$_2$, 1.0 MgSO$_4$, 24 NaHCO$_3$, 0.5 Na$_2$HPO$_4$, and 30 d-glucose equilibrated with 95% O$_2$-5% CO$_2$ (pH = 7.4). In cases where the [Ca$^{2+}$]$_o$ was decreased from 1.5 mM, the concentration of MgCl$_2$ was adjusted to maintain equal molar concentrations of divalent ions to eliminate the effects of reduced cation screening (Frankenhaeuser and Hodgkin 1957).

Recording and analysis

POPULATION RECORDINGS. Recordings of spinal motoneuron population activity in vitro were made with suction electrodes applied to the cut ends of spinal ventral roots and hypoglossal (XII) cranial roots. Extracellular recordings of population activity in medullary slice were made with suction electrodes placed in the XII motor pool or ventrolateral medulla (VLM) in the region of the pre-Bötzinger complex (pre-BöC). Signals were amplified, rectified, low-passed filtered, and recorded on computer by an analog–digital converter (Digidata 1322A, Axon Instruments, Foster City, CA) and data-acquisition software (Clampex). Mean values relative to control for the period of motoneuron discharge were calculated pre- and postdrug delivery. Results are expressed as mean ± SD and any differences were tested using paired/unpaired difference Student’s t-test; significance was accepted at values of P < 0.05.

WHOLE CELL RECORDINGS. Recording electrodes were fabricated from thin-wall borosilicate glass (1.5 mm external and 1.12 mm internal diameter; A-M Systems, Everett, WA). The pipette resistances were between 3 and 5 MΩ. The standard pipette solution contained (in mM): potassium gluconate, 130; NaCl, 10; CaCl$_2$, 1; 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid (BAPTA), 10; HEPES, 10; Mg-ATP, 5; Na-GTP, 0.3; pH 7.3 with KOH. In some experiments, CaCl$_2$ was omitted when examining intracellular Ca$^{2+}$ effects. Whole cell current-clamp recordings were performed with an npi SEC05LX amplifier (npi, Tamm, Germany). Liquid junction potentials were corrected before seal formation with the compensation circuitry of the patch-clamp amplifier. Data were digitized with an A/D interface (Digidata 1322A, Axon Instruments) and analyzed with the use of pCLAMP 9.0 (Axon Instruments). Recordings from neurons with a stable resting membrane potential ≤−45 mV and action potential amplitudes ≥50 mV were analyzed. XII motoneurones reside in a relatively homogeneous nucleus (<5% are interneurons) (Viana et al. 1993) and are easily identifiable under infrared differential interference contrast microscopy. They can be identified by location in the slice, characteristic morphology, and large-diameter somata.

Drugs

Stock solutions of drugs were prepared as concentrates. All drugs were added to the perfusate by switching to reservoirs containing the appropriate test solution. The following drugs were used: 6-cyano-7-nitroquinolin-2-3-dione (CNQX), bicuculline, strychnine, glycine, t-tubocurarine, riluzole, tetrodotoxin (TTX), tetraethylammonium chloride (TEA), dantrolene, carbenoxolone, doxyl-stearic acid, BAPTA, BAPTA acetoxymethyl ester (BAPTA-AM), dazocline maleate (MK-801), and suramin. Drugs were purchased from Sigma (St. Louis, MO) or RBI (Oakville, ON, Canada).

Optical imaging with a voltage-sensitive dye

Experiments were performed using spinal cord preparations isolated from E17 Wistar (n = 5) and Sprague–Dawley (n = 4) rat embryos (Saitama Experimental Animals Supply, Saitama, Japan). The preparations were labeled by incubating for 5–10 min in a solution containing 0.4 mg/ml of the voltage-sensitive dye merocyanine-rodhanide NK2761 (Hayashibara Biochemical Laboratories, Kankoh-Shiko Kenkyusho, Okayama, Japan). The preparations were then placed in a recording chamber with the ventral side facing up and visualized with an Eclipse E800 microscope (Nikon, Tokyo, Japan). The optical recording system used was similar to that described previously (Momose-Sato et al. 2001). In brief, bright-field illumination was provided by a 300-W tungsten–halogen lamp driven by a stable DC-power supply and incident light was collimated and rendered quasi-monochromatic with an interference filter with a transmission maximum at 699 ± 13 nm (half-width) (Asahi Spectra, Tokyo, Japan). The objectives (Plan Apo, ×4, 0.2 NA or ×2, 0.1 NA) and photographic eyepiece (×2.5) projected an image of the preparation onto a 34 × 34-element silicon photodiode matrix array mounted on the microscope. Changes in transmitted light intensity through the preparation were detected with the photodiode array and recorded with a 1,020-site optical recording system constructed in the Momose-Sato and Sato laboratory. Each pixel (element) of the array detected light transmitted by a square region (116 × 116 μm$^2$ using ×10 magnification) of the preparation. The optical signals were amplified (time constant of AC coupling = 3 s), passed through a low-pass filter (time constant = 470 μs), digitized with a 16-bit dynamic range, and sampled at 1,024 Hz. The recordings were made in single sweeps and no off-line filtering was used. $I_{\text{pre-staining}}/I_{\text{after-staining}}$ in the spinal cord averaged 34%, and regional differences were small. Thus the optical signals were presented as Δ$I_{\text{pre-staining}}/I_{\text{after-staining}}$ (the change in the transmitted light intensity divided by the incident light intensity). Spatiotemporal images were constructed using NeuroPlex software (Red Shirt Imaging LLC, Fairfield, CT). During optical recording, the spontaneous motor discharge on lumbar ventral roots (L1–L3) was recorded with suction electrodes. Signals, amplified with filters set at 0.08 Hz and 1 kHz, were digitally recorded at 4 kHz with an A/D converter (MacLab/8S, AD Instruments, Castle Hill, Australia), or fed into one channel of the A/D converter of the 1,020-site optical recording system.

RESULTS

Characterization of rhythmic activity

Electrophysiological recordings from spinal cord preparations: Fig. 1A illustrates the spontaneous rhythmic bursting in a spinal cord isolated from an E17 fetal rat maintained in standard in vitro conditions with 1.5 mM [Ca$^{2+}$]$_o$. As previously reported (Ren and Greer 2003), preparations isolated from fetal rats generated 4- to 12-s-duration rhythmic bursts occurring with an interburst interval of 2–3 min that occur bilaterally along the neuraxis (Table 1). Changing the bathing medium to one containing reduced [Ca$^{2+}$]$_o$ (zero [Ca$^{2+}$]$_o$) persisted in the right rhythms generated in 0.5 mM and zero [Ca$^{2+}$]$_o$. As previously reported (Ren and Greer 2003), preparations isolated from fetal rats generated 4- to 12-s-duration rhythmic bursts occurring with an interburst interval of 2–3 min that occur bilaterally along the neuraxis (Table 1). Changing the bathing medium to one containing reduced [Ca$^{2+}$]$_o$ was performed to diminish synaptic transmission. The rhythmic motor discharge was abolished immediately after the switch to the lower [Ca$^{2+}$]$_o$, perfusate. However, within 20 min, a very robust rhythmic motor discharge of 30- to 50-s duration with interburst intervals of 4–6 min reemerged despite the continued bathing of the preparation in zero [Ca$^{2+}$]$_o$ (Fig. 1D, Table 1). Similar rhythmic bursting was generated with a graded reduction of [Ca$^{2+}$]$_o$ (Fig. 1, B–D, Table 1). Consistent with past work (Ren and Greer 2003) the shorter-duration bursting seen in normal (i.e., 1.5 mM [Ca$^{2+}$]$_o$) bathing solution was blocked by a cocktail of receptor antagonists [CNQX (20 μM), MK-801 (50 μM), bicuculline (50 μM, free base), strychnine (20 μM), dazocline (40 μM)]. In contrast, the longer-duration rhythms generated in 0.5 mM and zero [Ca$^{2+}$]$_o$, persisted in the presence of the cocktail of receptor antagonists (Fig. 1, right). The spontaneous activity in 0.75 mM [Ca$^{2+}$]$_o$ was suppressed by the cocktail of antagonists in all six spinal cord preparations.
nonsynaptic spread of fetal rhythmic neuronal activity

FIG. 1. Spontaneous rhythmic discharge generated by the spinal cord preparations isolated from E17 rats. Simultaneous suction electrode recordings were made from C4 and L1 ventral roots. A–D, left: rectified and integrated suction electrode recordings of rhythmic discharge generated by an E17 preparation bathed in solution containing normal (1.5 mM) and reduced [Ca^{2+}]_o. Middle: details of individual bursts (* from left) on a shorter timescale with the dashed line indicating the relative onsets of cervical and lumbar ventral root bursts. Right: effects of adding a cocktail of receptor antagonists [6-cyano-7-nitroquinolinic acid (CNQX, 20 μM), MK-801 (50 μM), bicuculline (50 μM), strychnine (20 μM), turbeculurine (40 μM)] to the bathing medium. Quantification of the population data are presented in Table 1.

tested except for the occasional (approximately one per 20 min) long-duration bursts. All rhythmic bursting was blocked by bath application of TTX (1 μM).

All of the data presented are from preparations of ages E16–E18. Rhythmic activity on ventral or cranial roots in zero [Ca^{2+}]_o was not observed at older ages (E19–P4, n = 13) and was less consistent and of low amplitude at younger ages (E13–E15, n = 11). Zero [Ca^{2+}]_o-induced activity was also observed in all E17 Wistar rat spinal cords tested (n = 6) and there were no significant differences in the burst interval, duration, amplitude, and antagonist sensitivity between Sprague–Dawley and Wistar rats.

Multiple suction electrodes were used to record from different segmental levels of the isolated spinal cord preparations to examine the temporal relationship of bursting along the rostrocaudal axis. In bathing medium containing 1.5 or 1.0 mM [Ca^{2+}]_o, rhythmic activity consistently appeared on lumbar roots 200–400 ms before cervical roots (Table 1), which is consistent with results from past studies of E16–E18 preparations (Ren and Greer 2003). In contrast, the longer-duration bursting produced in reduced [Ca^{2+}]_o appeared with a nearly 6-s earlier onset on cervical versus lumbar ventral roots (Fig. 1, Table 1).

IMAGING OF SPINAL CORD PREPARATIONS WITH A VOLTAGE-SENSITIVE DYE. To further examine the spatiotemporal distribution of spontaneous rhythmic bursting, optical techniques with a voltage-sensitive dye in conjunction with electrophysiological recordings were used. Figure 2A shows the electrophysiological and optical recordings of spontaneous motor discharge produced in the lumbar spinal cord of an in vitro preparation bathed in 1.5 mM [Ca^{2+}]_o. The optical signal exhibited a smooth waveform that resembled the DC potential change of the electrical signal. Figure 2B shows the longer-duration bursting profile in zero [Ca^{2+}]_o solution. The action spectra of the voltage-sensitive dye NK2761 are such that the transmitted light intensity increases with depolarization in the range of 500–620 nm, decreases in the range of 640–750 nm, and is reduced to null near 630 nm (Momose-Sato et al. 1995). At 630 nm (Fig. 2B, bottom traces), the upward signal was not observed whereas the downward signal was detected. These results show that the initial upward signal corresponds to a dye-dependent absorption change (extrinsic signal) related to membrane depolarization and the downward component is an intrinsic optical change that may be attributable to cell swelling and the related shrinkage in the extracellular space associated with a large wave of depolarizing activity (Sato et al. 1997).

Figure 3 shows the spread of the optical signal along the rostrocaudal extent of the isolated spinal cord preparation in zero [Ca^{2+}]_o solution. Optical measurements were made from five locations along the neuraxis, whereas electrophysiological recordings were made from the lumbar spinal cord. Both the extrinsic and intrinsic signals were detected from the entire region of the spinal cord and they propagated slowly from the cervical to lumbar regions.

ELECTROPHYSIOLOGICAL RECORDINGS FROM SLICES OF SPINAL CORD AND MEDULLA. To examine the ability of cervical, thoracic, and lumbar cord sections to generate spontaneous activity in zero [Ca^{2+}]_o independently, the spinal cord was transected at the first (T1) and last (T13) thoracic levels in E17 preparations. The preparations were left for 30 min after transection before recordings. Spontaneous activity in zero [Ca^{2+}]_o solution was generated in each spinal section in all four preparations tested. Transections significantly prolonged the burst interval in each spinal cord section (P < 0.05). The interburst intervals were 8.1 ± 2.5, 13.3 ± 5.8, and 21.5 ± 8.5

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

**TABLE 1. Pattern and antagonist sensitivity of rhythmic activity generated in zero [Ca^{2+}]_o in E17 rat spinal cord preparations**

<table>
<thead>
<tr>
<th>[Ca^{2+}]_o (mM)</th>
<th>n</th>
<th>Interval, min</th>
<th>Duration, s</th>
<th>Coefficient of Variation of Burst Interval</th>
<th>Antagonist Action</th>
<th>Onset Delay, s</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>6</td>
<td>2.0 ± 0.91</td>
<td>8.7 ± 8.1</td>
<td>0.46 ± 0.07</td>
<td>Completely blocked</td>
<td>0.22 ± 0.09</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>4.3 ± 1.9*</td>
<td>22 ± 13.5*</td>
<td>0.44 ± 0.09</td>
<td>Completely blocked</td>
<td>0.34 ± 0.13</td>
</tr>
<tr>
<td>0.75</td>
<td>4</td>
<td>8.9 ± 3.4*</td>
<td>39 ± 14.3*</td>
<td>0.38 ± 0.08*</td>
<td>Partially blocked</td>
<td>−0.03 ± 0.12*</td>
</tr>
<tr>
<td>0.5</td>
<td>5</td>
<td>8.6 ± 2.8*</td>
<td>63 ± 8.3*</td>
<td>0.33 ± 0.06*</td>
<td>No effects</td>
<td>−6.7 ± 2.8*</td>
</tr>
<tr>
<td>0</td>
<td>9</td>
<td>4.4 ± 1.2*</td>
<td>42 ± 5.4*</td>
<td>0.27 ± 0.05*</td>
<td>No effects</td>
<td>−6.1 ± 2.7*</td>
</tr>
</tbody>
</table>

Values are means ± SD. *P < 0.05 compared with 1.5 mM [Ca^{2+}]_o.

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of a cocktail of receptor antagonists used to block synaptic transmission (Fig. 4B), but were blocked by TTX (1 μM).

Figure 5 shows the characteristics of an individual long-duration burst generated by the medullary slice in zero \([\text{Ca}^{2+}]_o\) on a shorter timescale using nerve root and whole cell recordings from XII motoneurons. Each long-duration burst consisted of about 25 short-duration (1-s) bursts with an interburst interval of 1–5 s. The resting membrane potential of XII motoneurons was \(-52.1 \pm 1.9\) mV in the normal \([\text{Ca}^{2+}]_o\) solution containing 9 mM \([\text{K}^+]_o\). After changing the solution to zero \([\text{Ca}^{2+}]_o\), there was a 10- to 15-mV membrane depolarization within 5 min followed by a return to \(-52.3 \pm 2.3\) mV within 15–20 min \((n = 7)\). XII motoneurons showed voltage-dependent bursting properties when bathed in zero \([\text{Ca}^{2+}]_o\), which was never observed in control solutions (Fig. 5C). All rhythmic bursting persisted in medullary slice preparations bathed in zero \([\text{Ca}^{2+}]_o\) in the presence of the cocktail of receptor antagonists.

**Ionic conductances involved in generation of rhythmic bursting generated in medullary slice preparations bathed in zero \([\text{Ca}^{2+}]_o\)**

Whole cell recordings from XII motoneurons in medullary slice preparations were performed to examine the ionic mech-

![Figure 2](image-url)  
**FIG. 2.** Electrophysiological and optical recordings of spontaneous motor discharge produced by spinal cord in vitro preparations isolated from an E17 rat. A: optical (top traces) and DC potential (bottom traces) recordings of spontaneous bursts from the lumbar spinal cord in vitro in control 1.5 mM \([\text{Ca}^{2+}]_o\) solution. Right traces: expanded time base of the left traces. Optical signal exhibited a smooth waveform, which resembled the DC potential change of the electrical signal. Magnitude of the optical signal represents the weighted optical average of the potential change and membrane area imaged onto each detector. B: spontaneous activity recorded from the lumbar spinal cord in zero \([\text{Ca}^{2+}]_o\) solution. Duration of the spontaneous activity was very long and the shape of the optical signal was quite different relative to that in 1.5 mM \([\text{Ca}^{2+}]_o\) solution. At 700 nm of the incident light (top traces), the optical signal showed a decrease in transmitted light intensity (upward deflection in the trace), which was followed by a large, long-lasting downward signal.

![Figure 3](image-url)  
**FIG. 3.** Propagation pattern of spontaneous activity in zero \([\text{Ca}^{2+}]_o\) solution. Pseudocolor images were obtained from the cervical (upper) and lumbar (lower) regions indicated with squares on the bottom right inset. Bottom left traces: optical signals detected from 5 regions indicated on the inset. Top traces: expanded time base of the left traces. All regions were capable of generating rhythmic bursting independently after they were separated by a lesion (data not shown). The rhythms generated by medullary slice tissue in zero \([\text{Ca}^{2+}]_o\) were not perturbed by the addition of a cocktail of receptor antagonists used to block synaptic transmission (Fig. 4B), but were blocked by TTX (1 μM).

Figure 5 shows the characteristics of an individual long-duration burst generated by the medullary slice in zero \([\text{Ca}^{2+}]_o\) on a shorter timescale using nerve root and whole cell recordings from XII motoneurons. Each long-duration burst consisted of about 25 short-duration (1-s) bursts with an interburst interval of 1–5 s. The resting membrane potential of XII motoneurons was \(-52.1 \pm 1.9\) mV in the normal \([\text{Ca}^{2+}]_o\) solution containing 9 mM \([\text{K}^+]_o\). After changing the solution to zero \([\text{Ca}^{2+}]_o\), there was a 10- to 15-mV membrane depolarization within 5 min followed by a return to \(-52.3 \pm 2.3\) mV within 15–20 min \((n = 7)\). XII motoneurons showed voltage-dependent bursting properties when bathed in zero \([\text{Ca}^{2+}]_o\), which was never observed in control solutions (Fig. 5C). All rhythmic bursting persisted in medullary slice preparations bathed in zero \([\text{Ca}^{2+}]_o\) in the presence of the cocktail of receptor antagonists.

**Ionic conductances involved in generation of rhythmic bursting generated in medullary slice preparations bathed in zero \([\text{Ca}^{2+}]_o\)**

Whole cell recordings from XII motoneurons in medullary slice preparations were performed to examine the ionic mech-
anisms associated with the zero [Ca\(^{2+}\)]\(_{o}\)-induced bursting. Persistent sodium currents (I\(_{\text{Nap}}\)) are thought to play an important role in intrinsic bursting properties of neurons and the generation of rhythmic discharge in zero [Ca\(^{2+}\)]\(_{o}\) (Butera et al. 1999; Darbon et al. 2004; Elson and Selverston 1997) and thus were examined in this study. The rhythmic bursting in zero [Ca\(^{2+}\)]\(_{o}\) was abolished in the presence of the nonspecific blocker of I\(_{\text{Nap}}\) riluzole (1–10 \(\mu\)M, Fig. 6A). The effects of riluzole (5 \(\mu\)M) took between 5 and 20 min and were reversible on washout (1–2 h) in 58% of preparations tested (\(n = 12\)). A voltage-clamp ramp protocol (30 mV/s) was used to demonstrate the presence of I\(_{\text{Nap}}\) and the blocking effect of riluzole in XII motoneurons (Fig. 6B). The peak I\(_{\text{Nap}}\) was 169 ± 75 pA (\(n = 6\)) at a membrane potential of −35 mV.

We evaluated the potential role of intracellular free Ca\(^{2+}\) in generating the long-duration bursts. Bath application of the cell-membrane–permeable agents BAPTA-AM (30 \(\mu\)M, Ca\(^{2+}\) chelating agent) and dantrolene (30 \(\mu\)M, intracellular Ca\(^{2+}\) release blocker) had no effects on zero [Ca\(^{2+}\)]\(_{o}\)-induced bursting frequency in spinal cord (\(n = 6\), Fig. 7A) or medullary slice preparations (\(n = 5\), data not shown). Bath applications of Ba\(^{2+}\) (100 \(\mu\)M, \(n = 5\)), Cd\(^{2+}\) (200 \(\mu\)M, \(n = 4\)), or TEA (10 \(\mu\)M, \(n = 5\)) did not suppress the zero [Ca\(^{2+}\)]\(_{o}\)-induced bursting, suggesting that neither Ca\(^{2+}\) nor K\(^{+}\) conductances are necessary for the induction of bursting (Fig. 7, B–D).

**Involvement of gap junctions**

Gap junctions are prominent among neurons and glia (Kiehn and Tresch 2002) in the fetal nervous system and thus we assessed their potential role in zero [Ca\(^{2+}\)]\(_{o}\)-induced bursting.

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**FIG. 4.** Spontaneous rhythmic discharge generated by medullary slice preparation isolated from an E18 rat. A, left: medullary slice preparation. Right: suction electrode recordings from the hypoglossal (XII) motor nucleus and the ventrolateral medulla (VLM). Inspiratory rhythmic discharge was generated by the isolated medullary slice preparation bathed in control solution (containing 9 mM K\(^+\)). Longer-duration rhythmic bursting emerged when bathed in zero [Ca\(^{2+}\)]\(_{o}\) solution. B: rhythmic activity generated in zero [Ca\(^{2+}\)]\(_{o}\), persisted in the presence of receptor antagonists (CNQX (20 \(\mu\)M), MK-801 (50 \(\mu\)M), bicuculline (50 \(\mu\)M), strychnine (20 \(\mu\)M), turbocurarine (40 \(\mu\)M)).

**FIG. 5.** XII motoneuron activity in an E18 medullary slice preparation bathed in zero [Ca\(^{2+}\)]\(_{o}\) solution. A: whole cell intracellular recording from XII motoneuron (top), suction electrode recordings from XII nerve root (middle), and VLM (bottom). B: details of the section of recording from A (*) on a shorter timescale showing that each long-duration rhythmic discharge consists of multiple individual short-duration bursts. C: further recordings of multiple individual short-duration bursts that demonstrate their voltage-dependent bursting properties in zero [Ca\(^{2+}\)]\(_{o}\). Voltage-dependent intrinsic bursting was suppressed by a 50-pA hyperpolarizing current and increased by a 40-pA depolarizing current and unaffected by addition of receptor antagonists.

**FIG. 6.** Effects of riluzole, a blocker of the persistent sodium current, on rhythmic discharge produced in zero [Ca\(^{2+}\)]\(_{o}\) solution. A, top trace: whole cell recording of a XII motoneuron. Bottom trace: suction electrode recording from the contralateral XII nucleus in an E18 medullary slice preparation. Bursting generated in zero [Ca\(^{2+}\)]\(_{o}\) solution was blocked by riluzole. Note that the neuron was capable of generating an action potential in the presence of riluzole in response to injected current (* shown in box). Rhythmic discharge in zero [Ca\(^{2+}\)]\(_{o}\) was partially recovered after 1 h of riluzole washout. B, left: inward current and negative slope region in response to an applied voltage-clamp ramp protocol over the interval −80 to 10 mV with a ramp speed of 30 mV/s. Voltage-insensitive leak current (I\(_{\text{leak}}\)) is characterized from the linear portion (between −80 and −65 mV) of the membrane I–V curve analyzed by a linear regression. Right: voltage-activated inward current (control) was extracted (data from left, solid curve) by subtracting the passive leak current (I\(_{\text{leak}}\)). Inward current and negative slope region are blocked by 5 \(\mu\)M riluzole.
A 5- to 10-min application of the nonspecific gap junction blocker carbenoxolone (100 μM) completely blocked bursting recorded from nerve roots in all spinal cord (E16, n = 2; E17, n = 3; E18, n = 2) and medullary slice (E17, n = 3; E18, n = 9) preparations bathed in zero [Ca\(^{2+}\)]\(_o\). The bursting recorded with whole cell recordings was abolished in three of seven XII motoneurons (E18 preparations) in the presence of carbenoxolone (100 μM, Fig. 8A). The action potential and synaptic drive amplitude were diminished in the remaining four XII motoneurons but bursting continued (Fig. 8B). Carbenoxolone (100 μM) did not significantly affect the resting membrane potentials in any of the XII motoneuron recordings. To further examine the role of gap junctions, doxyl-steaeric acid was tested. It has been proposed that 50 μM doxyl-steaeric acid blocks gap junctions without severely affecting neuronal properties (Su et al. 2001). Doxyl-steaeric acid blocked the zero [Ca\(^{2+}\)]\(_o\)-induced bursting on nerve roots in spinal cord (n = 3, data not shown) and medullary slice preparations (n = 7, Fig. 8C). However, whole cell recording in XII motoneurons demonstrated that XII motoneurons bursting continued in the presence of doxyl-steaeric acid.

Functional hemichannels also exist in isolation in the plasma membrane and can mediate the liberation of small molecules such as ATP and glutamate into the extracellular milieu with decreased extracellular [Ca\(^{2+}\)]\(_o\) (Hofer 2005). Thus we performed the additional experiment of including the nonspecific purinergic receptor antagonist suramin (1 mM) to the receptor antagonist cocktail to examine whether the release of ATP could have a role in the generation or transmission of the rhythms. There were no significant changes in the rhythmic bursts generated by E17 preparations bathed in zero [Ca\(^{2+}\)]\(_o\) with the addition of the suramin (n = 3).

**DISCUSSION**

Electrophysiological and optical recordings demonstrated that the fetal brain stem and spinal cord are capable of generating robust rhythmic neuronal discharge in the absence of synaptic drive. The rhythmic activity of in vitro preparations bathed in low or zero [Ca\(^{2+}\)]\(_o\) spread throughout the extent of the neuraxis. A role for intracellular Ca\(^{2+}\) fluxes was not supported because the rhythmic discharge was unaltered after applications of a Ca\(^{2+}\) chelator and suppressor of Ca\(^{2+}\) release from intracellular stores. Electrophysiological recordings examined the rhythmic discharge in motoneuron populations. It was clear that a perturbation of \(I_{\text{gap}}\) resulted in loss of rhythmic discharge at the motoneuron level. Further, the amplitude of recordings from synchronized motoneurons within ventral and cranial roots was diminished in the presence of nonspecific gap junction blockers, although bursting within individual motoneurons persisted.

These data are consistent with the hypothesis that there are additional mechanisms beyond gap junctions and synaptically.
mediated events to account for the strikingly widespread distribution of fetal rhythmic motor discharge. We propose that the following mechanisms are interacting and working in concert to transmit fetal rhythmic discharge.

1.) The primary mechanism for the generation and control of the timing of rhythmic bursting is by synaptically mediated transmission that involves acetylcholine, γ-aminobutyric acid, glycine, and excitatory amino acids, all of which have excitatory actions (Chub and O’Donovan 1998; Hanson and Landmesser 2003; Milner and Landmesser 1999; Nishimura et al. 1996; Ren et al. 2003). The overall balance of which neurotransmitter systems contribute to the generation and spread of rhythmic discharge changes with age. The extent to which neurons in distant regions extending from the lumbar spinal cord to the ventrolateral medulla are connected by a synaptic network is unknown, but the connectivity may not be so diffuse and ubiquitous to explain the full extent of transmission of rhythmic activity.

2.) There are gap junctions among homonymous motoneurons (Walton and Navarrete 1991) that transmit and synchronize activity. Further, there is evidence for some coupling among heterogeneous motoneuronal populations early in development (Bittman et al. 2004; Kiehn and Tresch 2002). The extent of gap junction coupling between nonmotoneuronal populations is unclear. However, there is no evidence for the extensive gap junction connectivity among neurons required to account for the spread of rhythmic activity along multiple axes of the spinal cord and brain stem.

3.) Data from this study indicate there are additional non-synaptic mechanisms that are capable of facilitating the diffuse spread of rhythmic neural activity throughout the neuraxis. These could include ephaptic coupling or field effects involving the generation of large extracellular currents and potential fields that alter the excitability of neighboring neurons and/or the synchronization of neuronal population activity (Bikson et al. 1999; Dudek et al. 1998; Jeffreys 1995).

We induced the generation of rhythmic activities with our experimental paradigm by altering extracellular [Ca$^{2+}$]o, and thus removing synaptic activity. The burst duration and inter-spike intervals were longer in zero [Ca$^{2+}$]o than in the [Ca$^{2+}$]o-induced rhythms compared with those generated under the control of synaptic events, although both rhythmic patterns are widespread throughout the neuraxis in fetal tissue. Comparable manipulations of bathing medium ionic composition have been used to generate rhythmic discharge in hippocampal slice in vitro preparations (reviewed in Dudek et al. 1998; Jeffreys 1995).

However, this is the first evidence for a rhythmic pattern emerging in the developing fetal spinal cord in the absence of synaptic transmission. Mechanisms underlying the generation and spread of rhythmic activity in hippocampal tissue include ephaptic and electrotonic interactions. The propagation rate of 0.5–10 mm/s observed for previous studies of the propagation of field effects in the absence of synaptic activity (Haas and Jeffreys 1984; Jeffreys and Haas 1982; Konnerth et al. 1984) were similar to those observed in electrophysiological and optical imaging recordings here. At a cellular level, $I_{\text{gap}}$ are enhanced in the hippocampal model and are necessary for the generation of after-depolarizing potentials and prolongation of individual bursts (Shuai et al. 2003), which is consistent with data in this study showing that hypoglossal motoneuron discharge and population spike were blocked by riluzole. Rhythmic bursting in hippocampal slices persisted in the presence of the gap junction blocker doxyl-stearic acid (Su et al. 2001). However, electrical coupling plays an important role in the synchronization and spread of neuronal activity among motoneuron populations when rhythmic discharge is induced pharmacologically in the neonatal spinal cord bathed in zero [Ca$^{2+}$]o (Tresch and Kiehn 2000). We found that rhythmic discharge persisted in XII motoneurons in the fetal medulla bathed in zero [Ca$^{2+}$]o in the presence of doxyl-stearic acid but the amplitude of synchronized motor axon activity recorded from the XII nerve root was greatly diminished. Our data do not provide direct information on the mechanisms underlying the generation and spread of rhythmic discharge among non-motoneuron populations within the in vitro preparations.

There is a clear age-dependent change in the characteristics of rhythmic motor patterns generated in the developing neuraxis. At early stages of development, modest action potential or synaptic activity can result in a significant reduction in [Ca$^{2+}$]i, and increased [K+]o, that would enhance ephaptic electrotonic interactions with ongoing synaptic and gap junction–mediated events (Cohen and Fields 2004; Kofuji and Newman 2004; Stringer 1998; Sykova et al. 1992). Here, we found that the propensity for the generation and spread of rhythmic activity post-E18 by nonsynaptic mechanisms in zero [Ca$^{2+}$]o was greatly diminished. That is also the developmental stage at which synaptically mediated events underlying the generation and spread of the rhythmic discharge are reduced and restricted to glutamate rather than redundant neurotransmitter systems (Ren and Greer 2003). At the cellular level, resting membrane potentials become more hyperpolarized and rheobase currents and chloride-mediated inhibition increase in late gestation (Martin-Caraballo and Greer 1999; McCobb et al. 1990; Rohrbaugh and Spitzer 1996; Wu et al. 1992; Xie and Ziskind-Conhaim 1995; Ziskind-Conhaim 1988). It is critical from a functional perspective that these multiple developmental changes occur to minimize the spatial spread of rhythmic discharge. In the rat, there is an emergence of respiratory and locomotor rhythms within the brain stem and spinal cord at E17–E18 (Greer et al. 1992; Kobayashi et al. 2001; Ozaki et al. 1996; Ren and Greer 2003) that require restricted and selected recruitment of neuronal circuitry. This is in contrast to earlier states where the cellular, synaptic, and network properties are such to maximize the successful generation and diffuse spread of general fetal rhythmic neuronal activity by multiple mechanisms.

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