RHYTHMIC NEURONAL DISCHARGE IN THE MEDULLA AND SPINAL CORD OF FETAL RATS IN THE ABSENCE OF SYNAPTIC TRANSMISSION

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ABSTRACT

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\textsuperscript{2+}]\textsubscript{o} 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 non-synaptically mediated conductances, potentially via 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.

Key words: rhythm generation, ephaptic, field effects, prenatal
INTRODUCTION

Episodes of spontaneous rhythmic activity are widespread in the developing vertebrate nervous system (Katz and Shatz, 1996; Nakayama et al., 1997; O'Donovan, 1999; Milner and Landmesser, 1999; Hanson and Landmesser, 2003; 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 (Dahm and Landmesser, 1991; Xie and Ziskind-Conhaim 1995; Spitzer and Ribera 1998; Hanson and Landmesser, 2004; Ba et al., 2004). The rhythmic activity commences from early stages of development when axons are path finding to their targets through to the late stages of gestation prior to 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 (Nishimaru et al. 1996; Chub and O'Donovan 1998; Milner and Landmesser 1999; Ren and Greer, 2003). Further, motoneurons are connected via gap junctions prenatally that facilitate the synchronization and spread of motor discharge (Tresch and Kiehn 2000; Bittman, 2004). 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 non-synaptic mechanisms for propagating neuronal activity that 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 (Jeffreys, 1995; Dudek et al., 1998). 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 anaesthetized with halothane (1.5% delivered in 95%O₂ and 5%CO₂) 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 upon delivery, the neuraxis was isolated from fetuses as previously described (Greer et al., 1992). **Spinal cord-brainstem preparations:** The spinal cord and brainstem was 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 1-2 lumbar segments were prepared. **Medullary slice preparations:** The brain stem-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 µm 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/minute, volume of the chamber 1.5 ml) with modified Kreb’s solution that contained (mM): 128 NaCl, 5.0 (brainstem-spinal cord preparations) or 9.0 (spinal cord or
medullary slice preparations) KCl, 1.5 CaCl₂, 1.0 MgSO₄, 24 NaHCO₃, 0.5 NaH₂PO₄, and 30 D-glucose equilibrated with 95%O₂ - 5%CO₂ (pH=7.4). In cases where the [Ca²⁺]₀ was decreased from 1.5 mM, the concentration of MgCl₂ was adjusted to maintain equal molar concentrations of divalent ions to eliminate the effects of reduced cation screening on neuronal excitability (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 preBötzing complex (preBötC). Signals were amplified, rectified, low-passed filtered and recorded on computer via an analogue-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 post-drug delivery. Results are expressed as mean ± standard deviation and any differences tested using paired/unpaired difference student's t test; significance was accepted at p values less than 0.05.

Whole-cell recordings: Recording electrodes were fabricated from thin wall borosilicate glass (1.5 mm external and 1.12 mm internal diameter purchased from A-M Systems, Inc., Everett, WA). The pipette resistances were between 3-5 MΩ. The standard pipette solution contained (mM): potassium gluconate (130), NaCl (10), CaCl₂ (1), 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA, 10), HEPES (10), Mg ATP (5), NaGTP (0.3); pH 7.3 with KOH. In some experiments, CaCl₂ 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 analog-to-digital 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 MNs reside in a relatively homogeneous nucleus (< 5% are interneurones) (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-nitroquinoxaline-2.3-dione (CNQX), bicuculline, strychnine, glycine, d-tubocurarine, riluzole, tetrodotoxin (TTX), tetraethylammonium chloride (TEA), dantrolene, carbenoxolone, doxyl-stearic acid, 1,2-bis(2-aminophenoxy)ethane-N,N,N’N’-tetraacetic acid tetrapotassium salt (BAPTA), BAPTA acetoxyethyl ester (BAPTA-AM), dizocilpine maleate (MK-801) and suramin. Drugs were purchased from Sigma (St. Louis, MO) or RBI (Oakville, ON).

**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 Co,
The preparations were labeled by incubating for 5-10 min in a solution containing 0.4 mg/ml of the voltage-sensitive dye merocyanine-rhodanine NK2761 (Hayashibara Biochemical Laboratories, Kankoh-Shikiso 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 Co, 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 1020-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 µm² using ×10 magnification) of the preparation. The optical signals were amplified (time constant of AC coupling = 3 sec), passed through an RC low-pass filter (time constant = 470 µsec), digitized with a 16-bit dynamic range, and sampled at 1024 Hz. The recordings were made in single sweeps, and no off-line filtering was used. $\frac{I_{\text{after staining}}}{I_{\text{before staining}}}$ in the spinal cord averaged 34%, and regional differences were small. Thus, the optical signals were presented as $\Delta I/I_{\text{after staining}}$ (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, USA). During optical recording, the spontaneous motor discharge on lumbar ventral roots (L1-L3) was recorded with suction electrodes. Signals were
amplified with filters set at 0.08 Hz and 1 kHz, and digitally recorded at 4 kHz with an analog-to-digital converter (MacLab/8S, AD Instruments, Castle Hill, Australia), or fed into one channel of the analogue-to-digital converter of the 1020-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\textsuperscript{2+}]\textsubscript{o}. As previously reported (Ren and Greer, 2003), preparations isolated from fetal rats generated 4-12 second duration rhythmic bursts occurring with an interburst interval of 2-3 minutes that occur bilaterally along the neuraxis (Table 1). Changing the bathing medium to one containing reduced [Ca\textsuperscript{2+}]\textsubscript{o} was performed to diminish synaptic transmission. The rhythmic motor discharge was abolished immediately after the switch to the lower [Ca\textsuperscript{2+}]\textsubscript{o} perfusate. However, within 20 minutes, a very robust rhythmic motor discharge of 30-50 second duration with interburst intervals of 4-6 minutes reemerged despite the continued bathing of the preparation in zero [Ca\textsuperscript{2+}]\textsubscript{o} (Fig. 1D, Table 1). Similar rhythmic bursting was generated with a graded reduction of [Ca\textsuperscript{2+}]\textsubscript{o} (Fig. 1B-D, Table 1). Consistent with past work (Ren and Greer, 2003) the shorter duration bursting seen in normal (i.e. 1.5 mM [Ca\textsuperscript{2+}]\textsubscript{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), turbocurarine (40 µM)). In contrast, the longer duration rhythms generated in 0.5 mM and zero [Ca\textsuperscript{2+}]\textsubscript{o} persisted in the presence of the cocktail of receptor antagonists (Fig. 1, right panels). The spontaneous activity in 0.75 mM [Ca\textsuperscript{2+}]\textsubscript{o} was suppressed by the cocktail of antagonists in all 6 spinal cord preparations.
tested except for the occasional (approximately 1 per 20 minutes) 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 SD 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 prior to 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 an approximate 6 second 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. Fig. 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 which resembled the DC potential change of the electrical signal. Fig. 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 (lower traces Fig. 2B), the upward signal was not observed while 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 due to cell swelling and the related shrinkage in the extracellular space associated with large wave of depolarizing activity (Sato et al., 1997).

Fig. 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 5 locations along the neuraxis while 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 minutes after transection prior to recordings. Spontaneous activity in zero [Ca$^{2+}$]$_o$ solution was generated in each spinal section in all 4 preparations tested. Transections significantly prolonged the burst interval in each spinal cord section ($P < 0.05$). The interburst intervals were 8.1±2.5 min, 13.3±5.8 min, and 21.5±8.5 min for cervical, thoracic, and lumbar sections, respectively, compared with 4.1±1.1 min when the spinal cord was intact.
Similarly, a robust rhythm was present on XII nerve roots of medullary slice preparations bathed in zero $[\text{Ca}^{2+}]_o$. Fig. 4A shows simultaneous recordings from the XII motoneuron pool and a region of the VLM containing the preBötC in an E18 medullary slice bathed control solution with normal and zero $[\text{Ca}^{2+}]_o$. The preBötC is composed of neurons located ventrolateral to the nucleus ambiguous that are thought to be important for respiratory rhythm generation (Smith et al., 1991). At this age, spontaneous inspiratory discharges consisting of short duration (approximately 700 ms) bursts are produced when slices are bathed in solution containing elevated $[\text{K}^+]_o$. The respiratory rhythm was abolished after changing to a zero $[\text{Ca}^{2+}]_o$ bathing medium. Within minutes, a very regular, long duration (50-100 seconds) bursting with an interburst interval of 3.3±0.8 min emerged. In intact slices, the rhythms produced in zero $[\text{Ca}^{2+}]_o$ in the XII and the preBötC were synchronized (Figs. 4, 5A-B). However, both 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).

Fig. 5 shows the characteristics of an individual long-duration burst generated by the medullary slice in zero $[\text{Ca}^{2+}]_o$ on a shorter time scale using nerve root and whole-cell recordings from XII motoneurons. Each long-duration burst consisted of approximately 25 short-duration (1 second) bursts with an interburst interval of 1-5 seconds. The resting membrane potential of XII motoneurons was -52.1±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 10-15 mV membrane depolarization within 5 minutes followed by a return to -52.3±2.3 mV within 15-20 minutes ($n=7$). XII motoneurons showed voltage-dependent bursting properties when bathed in $[\text{Ca}^{2+}]_o$ 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 mechanisms associated with the zero \([\text{Ca}^{2+}]_o\) -induced bursting. Persistent sodium currents (I_{Nap}) are thought to play an important role in intrinsic bursting properties of neurons and the generation of rhythmic discharge in zero \([\text{Ca}^{2+}]_o\) (Elson and Selverston, 1997; Butera et al., 1999; Darbon et al., 2004) and thus were examined in this study. The rhythmic bursting in zero \([\text{Ca}^{2+}]_o\) was abolished in the presence of the non-specific blocker of I_{Nap} riluzole (1-10 \(\mu M\), Fig. 6A). The effects of riluzole (5 \(\mu M\)) took between 5-20 minutes and were reversible upon wash-out (1-2 hrs) in 58% of preparations tested (n=12). A voltage-clamp ramp protocol (30 mV/s) was used to demonstrate the presence of I_{Nap} and the blocking effect of riluzole in XII motoneurons (Fig. 6B). The peak I_{Nap} was 169±75 pA (n=6) at a membrane potential of -35 mV.

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

Gap junctions are prominent amongst 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. A 5-10 minute application of the non-specific 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 3 of 7 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-stearic acid was tested. It has been proposed that 50 μM doxyl-stearic acid blocks gap junctions without severely affecting neuronal properties (Su et al., 2001). Doxyl-stearic 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-stearic 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 non-specific 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 brainstem and spinal cord is 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 as 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 \(I_{Nap}\) 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 non-specific 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 wide-spread 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 via synaptically mediated transmission that involves acetylcholine, GABA, glycine and excitatory amino acids, all of which have excitatory actions (Nishimaru et al., 1996; Chub and O’Donovan, 1998; Milner and Landmesser, 1999; Hanson and Landmesser, 2003; 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 via 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 amongst homonymous motoneurons (Walton and Navarrete, 1991) that transmit and synchronize activity. Further, there is evidence for some coupling amongst heterogeneous motoneuronal populations early in development (Kiehn and Tresch, 2002; Bittman et al., 2004). The extent of gap junction coupling between non-motoneuronal populations is unclear. However, there is no evidence for the extensive gap junction connectivity amongst neurons required to account for the spread of rhythmic activity along multiple axes of the spinal cord and brainstem. 3) Data from this study indicates 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 (Jeffreys, 1995, Dudek et al., 1998; Bikson et al., 1999).

We induced the generation of rhythmic activities with our experimental paradigm by altering extracellular \([\text{Ca}^{2+}]_0\) and thus removing synaptic activity. The burst duration and interspike intervals were longer in zero \([\text{Ca}^{2+}]_0\) induced rhythms compared to those generated under the control of synaptic events, although both rhythmic patterns are wide-spread 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 Jefferys, 1995; Dudek et al., 1998). 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 (Jefferys and Haas, 1982; Haas and Jefferys, 1984; Konnerth et al., 1984) were similar to those observed in electrophysiological and optical imaging recordings here. At a cellular level, \( I_{\text{Nap}} \) 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 amongst motoneuron populations when rhythmic discharge is induced pharmacologically in the neonatal spinal cord bathed in zero \([\text{Ca}^{2+}]_o\) (Tresch and Kiehn 2000). We found that rhythmic discharge persisted in XII motoneurons in the fetal medulla bathed in zero \([\text{Ca}^{2+}]_o\) 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 does not provide direct information on the mechanisms underlying the generation and spread of rhythmic discharge amongst 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 \([\text{Ca}^{2+}]_o\) and increased \([\text{K}^+]_o\) that would enhance ephaptic/electrotonic interactions with ongoing synaptic and gap-junction mediated events (Sykova et al, 1992; Stringer, 1998; Kofuji and Newman, 2004; Cohen and Fields, 2004).
Here, we found that the propensity for the generation and spread of rhythmic activity post-E18 by non-synaptic 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, rheobase currents and chloride mediated inhibition increases in late gestation (Ziskind-Conhaim, 1988; McCobb et al., 1990; Wu et al., 1992; Xie and Ziskind-Conhaim, 1995; Rohrbough and Spitzer, 1996; Martin-Caraballo and Greer, 1999). 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 brainstem 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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FIGURE LEGENDS

**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. Left panels in A-D show rectified and integrated suction electrode recordings of rhythmic discharge generated by an E17 preparation bathed in solution containing normal (1.5 mM) and reduced \([\text{Ca}^{2+}]_o\). The middle panel show details of individual bursts (* from left panel) on a shorter-time scale with the dashed line indicating the relative onsets of cervical and lumbar ventral root bursts. Right panels show the effects of adding a cocktail of receptor antagonists [CNQX (20 µM), MK-801 (50 µM), bicuculline (50 µM), strychnine (20 µM), turbocurarine (40 µM)] to the bathing medium. Quantification of the population data is presented in Table 1.

**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 are the expanded time base of the left traces. The optical signal exhibited a smooth waveform, which resembled the DC potential change of the electrical signal. The 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. The 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 (upper 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.

**Fig. 3** Propagation pattern of spontaneous activity in zero \([\text{Ca}^{2+}]_o\) solution. Pseudo-color images were obtained from the cervical (upper) and lumbar (lower) regions indicated with squares on the lower right inset. Lower left traces show optical signals detected from five regions indicated on the inset and electrical signals recorded from L2. Asterisks indicate the timing of appearance of the electrical signal. In the zero \([\text{Ca}^{2+}]_o\) solution, both the extrinsic and intrinsic signals were detected from the entire region of the spinal cord. They propagated slowly from the cephalic to caudal direction.

**Fig. 4** Spontaneous rhythmic discharge generated by medullary slice preparation isolated from an E18 rat. A) Left panel depicts medullary slice preparation. Right panels show 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 \([\text{K}^+]_o\)). Longer-duration rhythmic bursting emerged when bathed in zero \([\text{Ca}^{2+}]_o\) solution. B) Rhythmic activity generated in zero \([\text{Ca}^{2+}]_o\) persisted in the presence of receptor antagonists [CNQX (20 µM), MK-801 (50 µM), bicuculline (50 µM), strychnine (20 µM), turbocurarine (40 µM)].

**Fig. 5** XII motoneuron activity in an E18 medullary slice preparation bathed in zero \([\text{Ca}^{2+}]_o\) 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 time-scale 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\(^{2+}\)]\(_o\). The 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 is from a whole-cell recording of a XII motoneuron and the bottom trace is a suction electrode recording from the contralateral XII nucleus in an E18 medullary slice preparation. The 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\(^{2+}\)]\(_o\) solution partially recovered after 1 hour of riluzole washout. B) Left panel shows the 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\(_{leak}\)) is characterized from the linear portion (between -80 mV and -65 mV) of the membrane I-V curve analyzed by a linear regression. Right: The voltage-activated inward current (control) was extracted (data from left figure, solid curve) by subtracting the passive leak current (I\(_{leak}\)). The inward current and negative slope region is blocked by 5 µM riluzole.

**Fig. 7** Further characterization of the ionic conductances involved in rhythmic bursting in zero [Ca\(^{2+}\)]\(_o\) solution. All traces are rectified, integrated suction electrode recordings from L1 ventral root in spinal cord preparations isolated from E18 rats. A) Reducing intracellular concentration
with the Ca\(^{2+}\) chelator BAPTA-AM and the suppressor of intracellular Ca\(^{2+}\) store release dantrolene did not markedly effect rhythmic bursting in zero [Ca\(^{2+}\)]\(_o\) solution. B) The addition of Cd\(^{2+}\) to block the entry of any residual Ca\(^{2+}\) was without effect. C) Low concentrations of Ba\(^{2+}\) that block inward-rectifying K\(^+\) (Kir) currents did not affect the rhythmic discharge. D) The rhythm persisted in the presence of TEA that blocks outward K\(^+\) conductances.

**Fig. 8** The effects of gap junction blockers on rhythmic discharge produced in zero [Ca\(^{2+}\)]\(_o\) solution. Top traces are whole-cell recordings from XII motoneurons and bottom traces are suction electrode recordings from the contralateral XII nucleus in E18 medullary slice preparations. Right panel shows recordings after wash-out of gap junction blockers. A) Carbenoxolone (100 µM) abolishes both population rhythmic motor bursts recorded with extracellular electrode and the membrane oscillations in some XII motoneurons. B) Rhythmic membrane oscillations remained in a subpopulation of XII motoneurons in the presence of carbenoxolone (100 µM). C) The addition of the gap junction blocker doxyl-stearic acid (50 µM) diminished rhythmic motor bursts recorded with extracellular electrode but bursting within individual XII motoneurons recorded with whole-cell electrodes persisted.
Table 1: Pattern and antagonist-sensitivity of rhythmic activity generated in zero \([\text{Ca}^{2+}]_o\) in E17 rat spinal cord preparations

<table>
<thead>
<tr>
<th>[\text{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>Antagonists Action</th>
<th>Onset Delay (s)</th>
<th>C4 minus L1</th>
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<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>
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</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>
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<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>
<td></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>
<td></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>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SD; *p<0.05 compared with 1.5 mM [\text{Ca}^{2+}]_o.