Rhythmic Motor Activity in Thin Transverse Slice Preparations of the Fetal Rat Spinal Cord

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ABSTRACT

Networks generating locomotor-like rhythmic motor activity are formed during the last week of the fetal period in the rat spinal cord. We investigated the coordinated rhythmic motor activity induced in transverse slice preparations of the lumbar spinal cord taken from fetal rats as early as embryonic day (E) 16.5. In slices as thin as 100 µm, bath-application of 5-hydroxytryptamine (5-HT) induced rhythmic $[Ca^{2+}]_i$ elevations in motoneurons labeled with Calcium Green-1 dextran. The rhythmic $[Ca^{2+}]_i$ elevations were similar in frequency to that in the intact lumbar spinal cord, although there was no temporal correlation between the activity in the left and right sides of 100 µm slices. Such rhythmic $[Ca^{2+}]_i$ elevations were observed in the slices taken from all lumbar segments. Moreover, the rhythmic activity was abolished by simultaneous blockade of glutamate-, glycine- and GABA receptors, indicating that synaptic transmission mediated by these receptors is important for the generation of the rhythm in these slices. Synchronous rhythmic activity between the left-right sides was found in slices thicker than 200 µm taken from any segmental level of the lumbar spinal cord. In these preparations, commissural neurons were activated synchronously with ipsilateral motoneurons. These results indicate that the neuronal networks sufficient to generate coordinated rhythmic activity are contained in half of a single lumbar segment at E16.5. Such spinal cord slices are a promising experimental model to investigate the neuronal mechanisms and the development of rhythm generation in the spinal cord.
INTRODUCTION

Neuronal mechanisms generating locomotor-like rhythmic motor activity have been studied extensively using isolated spinal cord preparations. Bath-application of 5-hydroxytryptamine (5-HT) can induce rhythmic motor activity suitable to underlie locomotion in the spinal cords isolated from neonatal (Cazalets et al. 1992; Kiehn and Kjærulff 1996) and fetal rats (Iizuka et al. 1998; Nakayama et al. 2001). Studies using such preparations have revealed that 5-HT-induced rhythmic activity begins at embryonic day (E)14.5 (Nakayama et al. 2001) and that the rhythmic activity is synchronized between the left and right sides at the early developmental stages i.e. E14.5-16.5 (Iizuka et al. 1998). Such left-right synchronicity is mediated by excitatory connections via commissural neurons that couple the rhythm-generating networks on both sides (Nakayama et al. 2002a).

In the neonatal rat spinal cord, it has been shown that the rhythm-generating capability is distributed along the entire length of the lumbar spinal cord (Cowley and Schmidt 1997; Kjærulff and Kiehn 1996; Kremer and Lev-Tov 1997). Preparations consisting of only two or three segments are sufficient for generating the left-right coordinated rhythmic activity, although the upper lumbar segment seems to possess greater capability for generating a left-right alternating rhythm compared to lower lumbar segments (Kjærulff and Kiehn 1996; Kudo and Yamada 1987). However, little is known about the localization of the spinal neurons forming the neuronal networks responsible for rhythmic motor activity during the early fetal period. It also remains an open question whether the neuronal networks responsible for rhythmic motor activity
during the early developmental stages are distributed throughout the lumbar spinal cord or whether the basic units are localized in a few lumbar segments.

In the present study, using transverse slice preparations, we monitored the rhythmic oscillations in calcium concentration of motoneurons. These oscillations display a strong correlation with the rhythmic firing of these neurons during locomotor-activity in the isolated spinal cord preparations (Bonnot et al. 2002; O’Donovan et al. 1994). We show that synchronous rhythmic activity between the left and right sides can be observed in slices thicker than 200 µm taken from any segmental levels of the lumbar spinal cord in E16.5 rats. We also show that commissural interneurons, that have axons crossing in the midline, show rhythmic activity in these thin slices. A preliminary report has been presented in abstract form (Nakayama et al. 2002b).

METHODS

All experiments were performed with the approval of the Animal Research Committee of the University of Tsukuba, which operates in accordance with Japanese Governmental Law (No. 105). Experiments were performed on fetal Wistar rats aged E16.5. The lumbar spinal cord was obtained as previously described (Nishimaru et al. 1996) and maintained in ice-cold normal Krebs solution (composition in mM: NaCl, 118.4; KCl, 4.69; CaCl₂, 2.52; MgSO₄, 1.25; NaHCO₃, 25.0; KH₂PO₄, 1.18; D-glucose, 11.1) saturated with 95% O₂ and 5% CO₂ (pH 7.3-7.4 at room temperature, 24-26 °C). After ventral laminectomy, small crystals of Calcium Green-1 dextran, a calcium-sensitive dye, were placed on the ventral roots on left and right sides of the lumbar
spinal cord for retrograde labeling of motoneurons on both sides, following the methods described by O’ Donovan et al. (1994). In other preparations, the crystals were placed on the ventral roots on one side of the lumbar spinal cord and in the ventral funiculus on the opposite side for retrograde labeling of both motoneurons and commissural neurons on the same side. After incubation for 5-10 h, the spinal cord was embedded into 4% agarose solution and cooled on ice. The embedded spinal cord was glued to the stage of a vibratome (WPI, Worcester, MA), and was cut into transverse 100-500 µm thick slices. The slices from various segmental levels of the lumbar spinal cord were used in experiments. After incubation for 1 h, the transverse slice was placed in the recording chamber. We visualized Calcium Green-labeled neurons using an upright microscope (BX51WI; Olympus, Tokyo, Japan) fitted with a 100W short-arc mercury lamp, optical filters (excitation filter, 475-495 nm; emission filter, 515-550 nm), and water-immersion objectives (10×, 0.30NA; 40×, 1.15NA; Olympus). Fluorescence images were captured with an intensified CCD camera (ORCA-ER; Hamamatsu photonics, Japan), the fluorescence intensity was imaged using an image acquisition and analysis system for videomicroscopy (Aquacosmos; Hamamatsu photonics, Japan). Images were acquired at a frequency of 3 Hz. Data on calcium imaging are given as mean ± SE. Ventral root recordings in the intact spinal cord and measurements of the frequency of the rhythmic motor bursts were carried out as previously described (Nakayama et al. 2001). 5-HT, kynurenate, bicuculline (Sigma, USA), and strychnine (Wako Chemicals, Japan) were dissolved in the perfusate from stock solutions.

The coupling strength between the two regions, left and right side motoneurons or
motoneurons and commissural neurons, was analyzed using circular statistics (Batschelet, 1981). The phase values of 10-20 $[\text{Ca}^{2+}]_i$, elevation onsets on one region from each slice preparation were calculated with regard to onsets on the other region and the values were plotted on a circle representing the interval of possible phases from 0 to 1. The phase values 0 and 1 are equivalent and reflect synchrony, whereas 0.5 is equivalent to alternation. The mean phase and the coupling ratio ($r$) that describes the concentration of phase values around the mean were shown by the direction and the length of the vector originating from the center of the circle. If $[\text{Ca}^{2+}]_i$ elevations on the two regions are strongly coupled, then phase values would be expected to be highly concentrated around the mean phase. The coupling was considered significant when the Rayleigh test, which determines whether the concentration $r$ is sufficiently high to state that coupling was present (Batschelet, 1981), resulted in a $p$ value < 0.001.

RESULTS

*Left-right coordination of rhythmic calcium elevation in the transverse slice*

Left and right motoneuron pools in transverse slices were specifically labeled (Fig. 1A) by placing small crystals of Calcium Green-1 dextran on the left and right lumbar ventral roots. Fluorescence intensity, which indicates intracellular free Ca$^{2+}$ concentration ($[\text{Ca}^{2+}]_i$), in these labeled neurons was measured. Bath-application of 5-HT (1 µM) induced a tonic elevation of $[\text{Ca}^{2+}]_i$ followed by rhythmic elevations in $[\text{Ca}^{2+}]_i$, even in slices as thin as 100 µm (Fig. 1B). Such elevations in $[\text{Ca}^{2+}]_i$ were completely abolished by simultaneous application of kynurenate (an ionotropic
glutamate receptor antagonist; 4 mM) and strychnine (a glycine receptor antagonist; 2 µM) and bicuculline (a GABA<sub>A</sub> receptor antagonist; 5 µM) \((n = 3; \text{ Fig. } 1C)\). These results indicate that network activity mediated by these receptors is important for generation of elevations in \([\text{Ca}^{2+}]_i\) in motoneurons. 5-HT-induced rhythmic activity was observed in preparations taken from both rostral (L1-L3) and caudal (L4-L6) segments. Figure 1D shows representative rhythmic \([\text{Ca}^{2+}]_i\) elevations observed in slices of 500 µm in thickness taken from L2 and L5 segments. The frequency of the rhythm was 0.028-0.170 Hz (mean 0.116 ± 0.007 Hz; \(n = 24\)) in slices taken from rostral segments and 0.042-0.164 Hz (mean 0.083 ± 0.007 Hz; \(n = 22\)) in ones from caudal segments. The frequency of the rhythmic motor activity in the lumbar ventral roots in the intact spinal cord preparations was 0.061-0.122 Hz, (mean 0.090 ± 0.004 Hz; \(n = 19\), Nakayama et al. 2001) and was not different from that observed in slice preparations \((p = 0.22, \text{ Student’s } t\text{-test})\). These results indicate that the rhythmic \([\text{Ca}^{2+}]_i\) elevations observed in slices are generated by a similar neuronal mechanism to the rhythmic motor activity recorded in intact spinal cord preparations.

Although rhythmic activity could be induced in slice preparations as thin as 100 µm, there was no coupling between the rhythmic \([\text{Ca}^{2+}]_i\) elevations in the left and right motoneuron pools in these reduced preparations \((n = 5; \text{ Fig. } 1E)\). The left-right synchronous rhythmic \([\text{Ca}^{2+}]_i\) elevations could be observed in slice preparations thicker than 200 µm taken from any segmental levels of the lumbar spinal cord (Fig. 1, \(F\) and \(G\)). In 50 % of 200 µm-thick slices \((n = 10)\), the rhythmic \([\text{Ca}^{2+}]_i\) elevations were synchronous between motoneurons on the two sides. Moreover, in all preparations of
300 µm thickness (n = 13) and in 67% of 400 µm-thick slices (n = 6) and in 88% of 500 µm-thick slices (n = 8), the rhythmic [Ca\(^{2+}\)] elevations were synchronous. These results suggest that the pathway mediating the left-right coordination of the rhythmic activity was preserved even in 200 µm-thick slices, which is about half of the size of a single segment in the lumbar spinal cord (400-500 µm at E16.5; data not shown). We were able to observe such bilaterally synchronous activity in slices taken from both rostral and caudal segments, indicating that there is little difference in the capability of the left-right coordination along the rostro-caudal spinal axis.

In these thin slices, we examined the pathway and neurotransmitter mediating the left-right coordination of the rhythmic motor activity. After lesioning of the ventral commissure, the rhythmic [Ca\(^{2+}\)] elevations on the two sides became uncoupled (n = 3; Fig. 2, A and B), indicating that the ventral commissure is the crucial pathway for the coordination of rhythmic activity between left and right sides in these slices. Since the GABA\(_A\)-receptor-mediated synaptic transmission has been shown to be essential for the left-right coordination of the rhythmic motor activity in the intact spinal cord preparation at E15.5 (Nakayama et al. 2002a), we examined the effects of blocking GABA\(_A\) receptors on the left-right synchronized rhythm. The rhythmic [Ca\(^{2+}\)] elevations in the left and right motoneuron pools (Fig. 2C) were reversibly uncoupled by bath-application of bicuculline (2-10 µM) (n = 5; Fig. 2, D-F), indicating that synaptic transmission via GABA\(_A\) receptors is important for the connection of the rhythmic activity between the left and right sides in a single segment as well. Strychnine (2 µM), on the other hand, failed to disrupt the left-right synchronicity of the rhythmic activity
suggesting that glycinergic synapses are less involved than GABAergic ones in left-right coordination at this age.

**Rhythmic activity of commissural neurons in the slice preparation**

Since the ventral commissure is the crucial pathway for the left-right synchrony in the slice preparation, we examined the activity of commissural neurons during rhythmic motoneuronal discharge in this preparation. The labeled commissural neurons were mainly located in the ventromedial region in the transverse plane that corresponds to our previous report (Nakayama et al. 2002a). In all 200 µm \( (n = 4) \) and 500 µm-thick slices \( (n = 4) \), the \([Ca^{2+}]_i\) elevations in the commissural neurons were synchronous with the rhythmic \([Ca^{2+}]_i\) elevations in motoneurons (Fig. 3, A and B). Moreover, after splitting the slice in the midline, the synchronicity of the rhythmic activity in unilaterally located motoneurons and commissural neurons was preserved \( (n = 6; \text{ Fig. } 3C) \). These results suggest that in these slice preparations, commissural interneurons receive signals from the ipsilateral rhythm-generating network that are in phase with the signals sent to motoneurons.

In these slice preparations, we removed the dorsal half of the spinal cord to see if neurons in the ventral region alone are capable of generating the rhythm. The rhythmic activity could still be observed in the motoneurons \( (n = 6) \) and the commissural neurons \( (n = 3) \) after removing the dorsal part of the slice. Moreover, the phase relationship between the motoneurons and the commissural neurons after the lesion was unchanged before and after the lesion (Fig. 3D). These results indicate that the neuronal population
generating the rhythmic activity is located in the ventral part of the spinal cord.

DISCUSSION

In the present study, we show that a network of spinal neurons contained in slice preparations as thin as 100 µm is capable of generating rhythmic activity at this early fetal stage of development in the rat. Left-right connection of these networks was preserved in 200-300 µm-thick slices, which are thinner than a single lumbar segment. Moreover, such rhythmic activity was observed in slices taken from any segmental level in the lumbar spinal cord and the nature of the activity was extremely similar to that of the rhythmic motor activity obtained from whole lumbar spinal cord at the same stage. These results suggest that rhythm-generating networks are distributed throughout the rostro-caudal axis of the lumbar spinal cord, but comprise of relatively small groups of neurons. It is likely that these networks are connected with each other in the intact lumbar cord to generate synchronized rhythmic motor activity between rostral and caudal motoneurons during this early developmental period (Iizuka et al. 1998).

During fetal period, the spatial pattern of the 5-HT-induced rhythmic activity between motoneurons located in the rostral and caudal segment undergoes a drastic change from synchronicity to alternation. Furthermore, it has shown in studies using the neonatal rat that the upper lumbar region has a greater ability to generate a locomotor rhythm than the lower one, suggesting a rostro-caudal gradient in the distribution of the network in the lumbar spinal cord (Cazalets et al. 1995; Kjærulff and Kiehn 1996). In the present study, we showed that both rostral and caudal lumbar segments have the
capability to generate the synchronous rhythmic activity, similar to the whole lumbar spinal cords during early developmental period.

We have also shown that the ventral region of the slice alone is capable of generating rhythmic activity similar to that observed in the whole slice preparation. This is consistent with previous studies showing that the isolated ventral half of the spinal cord can generate coordinated rhythmic activity in the neonatal rat (Bracci et al. 1996; Kjærulff and Kiehn 1996) and in embryonic chick (Ho and O’Donovan 1993). Our results indicate that neurons located in the ventral part of the spinal cord are crucial elements of the rhythm-generating network from a very early stage. Since these slice preparations contain a much smaller neuronal population compared to the intact lumbar cord, they may be greatly advantageous to investigate the neuronal mechanisms of rhythm activity in the fetal spinal cord. In particular they may assist in the identification of interneurons constituting the rhythm-generating network.
DISCLOSURES

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FIGURE LEGENDS

FIG. 1. Rhythmic [Ca\textsuperscript{2+}], elevations in transverse slices of the lumbar spinal cord. A: a representative image of the Calcium Green-labeled left and right motoneuron pools in a thin slice preparation. Each motoneuron pool is surrounded by the broken line. Mean fluorescence intensity (F) in the area surrounded by the broken line was measured and the fluorescence change (ΔF/F) was traced. B: The onset of the 5-HT-induced [Ca\textsuperscript{2+}], elevations. Fluorescent videomicrographs of the slice during [Ca\textsuperscript{2+}], elevations are shown in the lower panel. C: Effects of bath-application of 4 mM kynurenate and 2 µM strychnine and 5 µM bicuculline on 5-HT-induced rhythmic fluorescence change. The rhythmic fluorescence change, indicating rhythmic [Ca\textsuperscript{2+}], elevation, induced by bath-application of 1 µM 5-HT (top trace) was blocked by application of these antagonists (middle trace) in a reversible manner (bottom trace). D: 5-HT-induced rhythmic [Ca\textsuperscript{2+}], elevations in the motoneuron pool in slices taken from L2 and L5 segments of a spinal cord. E: 5-HT-induced rhythmic [Ca\textsuperscript{2+}], elevations in the left and right motoneuron pools in the slice of 100 µm in thickness. The circular plot shows phase lags between the left and right sides. F: 5-HT-induced rhythmic [Ca\textsuperscript{2+}], elevations in the left and right motoneuron pools in the slice of 300 µm thickness. G: Coupling ratio (r) between the left and right motoneuron pools in transverse slice of each thickness.

FIG. 2. Neuronal mechanisms mediating the left-right coordination. A and B: Effects of lesion of the ventral commissure in the transverse slice. C, D and E: Effects of bath-application of bicuculline on left-right synchronized rhythmic [Ca\textsuperscript{2+}], elevations in
transverse slice. Synchronicity between left and right sides (C) was uncoupled by application of 10 µM bicuculline (D). E: recovery from effects of bicuculline. F: Mean coupling ratio in 5 preparations. G, H and I: Effects of bath-application of 2 µM strychnine on left-right synchronized rhythmic \([\text{Ca}^{2+}]\), elevations.

FIG. 3. Rhythmic activity of commissural neurons in the slice preparation. A: Calcium green fluorescence intensity on motoneurons (Mn) and ipsilateral commissural neurons (CN) in a transverse slice 500 µm in thickness during bath-application of 1 µM 5-HT. The circular plot shows phase lags between the motoneurons and commissural neurons. Lower right panel of A shows low-power image of Calcium Green-labeled motoneurons and commissural neurons in the transeverse slice. B: coupling ratio between motoneuron pools and commissural neurons in each transverse slices 200 or 500 µm in thickness. C and D: Calcium Green fluorescence intensity on the unilateral motoneurons and commissural neurons after cutting a hemicord (C) of the transverse slice, and after removing the dorsal part of the slice (D). The lower panel of D shows light microscopic images of a whole slice preparation (left) and isolated ventral quadrant of the slice (right). The fluorescence intensity (top trace of D) was measured in this isolated ventral quadrant preparation. Rhythmic activity could be induced until at least 3 hours after the lesion.
Figure 1
Figure 2

A. Control

B. Ventral commissure cut

C. Control

D. Bicuculline

E. Wash

F. Coupling ratio ($r$)

G. Control

H. Strychnine

I. Coupling ratio ($r$)
Figure 3