J Physiol Sci (2010) 60:303–307
DOI 10.1007/s12576-010-0091-1

SHORT COMMUNICATION

Spontaneous respiratory rhythm generation in in vitro upper cervical slice preparations of neonatal mice

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Received: 28 December 2009 / Accepted: 1 April 2010 / Published online: 24 April 2010

Abstract Isolated upper cervical slice preparations were prepared from neonatal mice to examine whether spontaneous respiratory activity could be generated in the preparations. By using brainstem-spinal cord preparations, we first recorded from the cervical C1–C2 and C4 ventral roots rhythmic bursts which were synchronized with respiratory burst activity of the hypoglossal (XIIth) nerve. Following transection just above the C1 segment, smaller and slower rhythmic bursts still persisted in the C1/C2 ventral roots and these were synchronized with those in the C4 ventral root. The present result, that a bursting rhythm remained in the C1/C2 slices, suggests that the spinal neuronal circuit for generating respiratory rhythm is localized in the upper cervical segments which contain upper cervical inspiratory neurons.

Keywords Upper cervical inspiratory neuron (UCIN) · Upper cervical slice · Neonatal mouse · Respiratory nerve activity

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Introduction

It has been proposed that respiratory rhythm generators in mammals consist of at least two distinct generators: the pre-Bötzinger complex (preBötC) [1, 2] and the parafacial respiratory group (pFRG) [3, 4] localized in the rostro-ventrolateral medulla. Findings obtained from neonatal in vitro preparations suggest that respiratory rhythm may be generated by pacemaker-like neurons in the two regions. The medullary respiratory neuron groups, triggered by the pacemaker-like neurons, may control the breathing rhythm, respiratory movement, and other respiratory-related muscles. A different group of localized respiratory neurons has been found in the C1/C2 segments of the cervical cord in the cat [5]. These propriospinal respiratory neurons, designated as the upper cervical inspiratory neurons (UCINs), receive inputs from medullary respiratory neurons and project to lower spinal segments [6–11]. The UCINs have also been found in rats and other rodents [12]. The existence of some excitatory monosynaptic and paucisynaptic connections to the ipsilateral phrenic motoneurons has been confirmed in cats and rats [13, 14]. It is assumed therefore that, together with the medullary respiratory neurons, the UCINs contribute to the formation of respiratory rhythm and pattern [15, 16]. To date, the role of the UCINs in spinal respiratory rhythm generation has not been clarified [17–19]. Therefore, it is necessary to investigate

whether the UCINs are involved in generation of the spinal respiratory rhythm [17].

In this study, we focused on the issue of spinal respiratory rhythm generation in isolated cervical slice preparations of mice. If a spontaneous respiratory rhythm could be generated in slice preparations which contain the putative UCINs, then involvement of the UCINs in generating a respiratory rhythm could be considered. Therefore, we attempted to record respiratory burst activity from cervical slice preparations. A part of the present study has been previously published in abstract form [20].

Materials and methods

Brainstem spinal-cord preparations

Neonatal (aged 1–5 days) ICR mice were used in this study. All of the surgical and animal care methods conformed to the Guidelines for the Use of Animals of the International Brain Research Organization. The experimental protocol was approved by the Sapporo Medical University Animal Care and Use Committee (Sapporo, Japan). The brainstem was cut approximately 0.5 mm rostral from the obex level. Brainstem spinal-cord sections which included hypoglossal (XIIth) nerve rootlets and the C1–C4 ventral roots were prepared in artificial cerebrospinal fluid (ACSF) at room temperature (Fig. 1a). The ACSF, with pH 7.4, consisted of (in mM) 123 NaCl, 3.5 KCl, 2.4 $CaCl_2$, 1.3 $MgSO_4$, 20 $NaHCO_3$, 0.64 NaH_2PO_4, and 30 D-glucose, gassed with 95% O_2, 5% CO_2. The preparations were transferred to a recording chamber and superfused with the ACSF with a flow rate of 2–4 ml/min at 26°C. The K^+ concentration was increased to a total of 5–6 mM in the ACSF for maintenance of respiratory activity.

Recordings, and transections to make upper cervical slice preparations

Extracellular glass-suction electrodes applied to the hypoglossal (XIIth) nerve rootlets and the C1–C4 ventral roots enabled recordings of rhythmic respiratory bursts from the hypoglossal nerve rootlets as output activity of the PBC, C1–C2 motoneuron bursts, and C4 phrenic activity (Fig. 1). The signals were amplified and filtered (band pass 10–3 k Hz) by a DAM 80 (World Precision Instruments, USA), digitized using an AD converter (DigiData 1200; Axon Instruments, Foster City, CA, USA), and were stored on a Windows computer. After confirming the rhythmic respiratory bursts, a brainstem spinal-cord preparation was transected, at the upper border of the C1 segment and lower border of the C2 segment, with a razor blade to make an upper cervical slice preparation (thickness approximately 1.5 mm; Fig. 1).

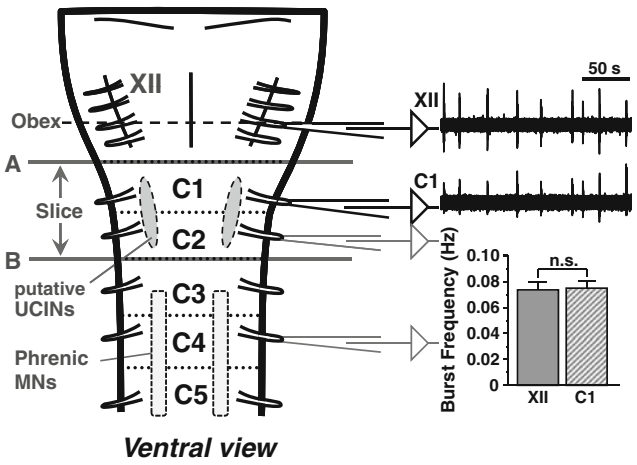

Fig. 1 Schematic illustrations of recordings of respiratory activity in the upper cervical slice preparations. Spontaneous bursts were recorded from the hypoglossal (XIIth) nerves and the ventral roots of C1–C4 in the brainstem spinal-cord preparations by using suction electrodes. Rhythmic C1 bursting activity was established as respiratory in nature by synchronized XIIth nerve activity (*upper right*). Significant differences between the bursts frequencies of the XIIth nerve and the C1 roots were not observed ($n = 8$, paired t tests; *lower right*). *Horizontal lines A* and *B* on the diagram of the brainstem spinal-cord preparation indicate the transection levels which were used to make the upper cervical slice preparations. The putative upper cervical inspiratory neurons (*UCINs*) and the phrenic motoneurons (*Phrenic MNs*) are located in the C1–C2 and the C3–C5 levels, respectively

Statistical analysis

The effects of the each transection were quantified as a change in the frequencies of the rhythmic respiratory bursts. A paired t test was used for statistical analysis of the data, using a significance level of $P < 0.05$. The data are listed as the mean percentage ± standard error (SE) throughout.

Results

In the present study, we confirmed that in vitro brainstem-spinal cord preparations generated spontaneous rhythmic respiratory burst activity in the spinal ventral roots (C1/C2 and C4), which was synchronous with that of the hypoglossal (XIIth) nerve rootlets. Significant differences were not observed between the XIIth and C1 burst frequencies (XII, 0.074 ± 0.006 Hz; C1, 0.075 ± 0.006 Hz; $n = 8$, paired t test; Fig. 1 right).

After confirming synchronized rhythmic respiratory bursts in the C1/C2 and C4 ventral roots (Fig. 2a), transection at the upper border of C1 (Fig. 1, transection "A")

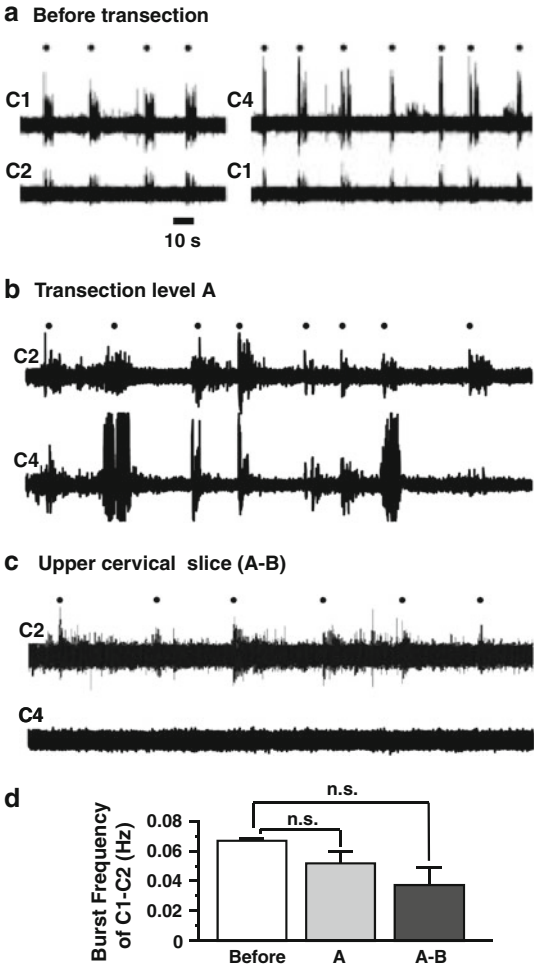

Fig. 2 Respiratory rhythm bursts in the brainstem spinal-cord preparation and the upper cervical slice preparations. **a** Rhythmic respiratory bursts, marked by *dots*, were obtained from the C1, C2, and C4, ventral roots in the brainstem spinal-cord preparation. Synchronized rhythmic respiratory bursts were recorded from the C1–C4 ventral roots. These bursts were synchronized with XIIth nerve activity. **b** After transection A, at the upper border of C1, the respiratory bursts persisted but with lower and irregular burst frequencies. **c** After transection B, at the lower border of C2 (shown in Fig. 1), respiratory bursts in the C2 roots were observed with lower frequencies, but the burst activity in the C4 ventral root was eliminated. **d** A summary of the respiratory burst frequencies before and after transections does not show any significant difference between each of the groups [*Before* before transection, *A* after transection at level A, *A–B* upper cervical slice (A–B); $n = 5$ each, paired t tests]

was performed. Following transection at the level of A, the respiratory bursts in the C4–C2 ventral root which showed a rapid onset–slowly decrementing burst pattern still persisted, but with lower and irregular burst frequencies (Fig. 2b). Secondly, we performed a transection at the lower border of C2 (Fig. 1, transection "B") to make an upper cervical slice preparation ("A–B slice"). In the A–B slice preparation, respiratory bursts in the C2 ventral roots still remained with lower frequencies (Fig. 2c). But the burst activity in the C4 ventral root was completely eliminated after transection "B" and was not observed thereafter. The SE of the mean frequency increased significantly by each transection, indicating the increase of irregularity of the burst activity (Fig. 2d). The burst frequencies in C1/C2 were decreased by each transection, but there were no significant differences before and after each transection, as shown in Fig. 2d (before, 0.067 ± 0.001 Hz; transection A, 0.052 ± 0.008 Hz; A–B slice 0.037 ± 0.012 Hz, $n = 5$ each, paired t tests). Spontaneous burst activity in the C1/C2 ventral roots occurred shortly ($\sim$30 min) after transection and remained stable in the cervical slice preparations for about 30 min and gradually deteriorated to cessation in 1–2 (range 0.5–3) h.

Discussion

The present work revealed that in vitro brainstem-spinal cord preparations of neonatal (aged 1–5 days) mice generated spontaneous rhythmic respiratory burst activity in the cervical ventral roots of C1/C2 and C4. This activity was synchronous with that of the hypoglossal (XIIth) nerve rootlets. Previous work in neonatal (aged 0–4 days) rats demonstrated the respiratory motor output from the cervical ventral roots of C1 and C4 [21]. Respiratory bursts recorded from the C3–C5 ventral roots are considered to be derived from phrenic motor output. The respiratory bursts in C1/C2 may originate, at least in part, from activity of the putative mouse UCINs localized in the upper cervical (C1/C2) segments.

It is known that in cats and rodents UCINs form a column of neurons localized near the lateral edge of the intermediate gray matter [8, 9]. The UCINs in cats receive excitatory inputs from two groups of medullary inspiratory neurons: the ventral respiratory group (VRG), and the dorsal respiratory group (DRG) [7]. Furthermore, previous workers demonstrated that in cats and rats some UCINs make monosynaptic and paucisynaptic connections to phrenic motoneurons [13, 14]. From these findings, it is assumed that UCINs may play a role as propriospinal respiratory interneurons. As for the other possible roles of UCINs, they may contribute to the coordination of respiratory motoneurons for non-respiratory functions, such as vomiting [22], and because some UCINs also send descending axons to the lower (thoracic and lumbar) spinal segments, they may function as propriospinal interneurons for the voluntary motor and/or locomotor system [23].

In in vitro brainstem-spinal cord preparations of neonatal rats, previous workers [24–26] have identified a spinal respiratory generator located mainly in the cervical C5 segment, and partially extending into the C4 and C6 segments. In their investigation, long-lasting spinal

inspiratory bursts were obtained by pharmacological activation, although more recent study indicated that α1-adrenergic receptor-mediated slow rhythmic bursts were unlikely to be related to the respiratory system [27]. In contrast, in our work which used isolated mouse preparations, spinal respiratory bursts were recorded in the C1/C2 ventral roots without any pharmacological activation.

Although no evidence is available, it may be postulated that putative UCINs with pacemaker-like properties connect directly, or indirectly, via interneurons to motoneurons in the C1/C2 ventral horns. With respect to the functional significance of putative UCINs, the C1/C2 ventral roots and the hypoglossal XIIth nerve roots jointly innervate the respiration-related muscles: the infrahyoid and suprahyoid muscles, respectively. In a recent study which used isolated brainstem-spinal cord preparations of neonatal rats, a novel respiratory neuron group was located around the ventral edge of the ventral horn of the C1/C2 segments [28]. This neuron group is considered to be distinct from UCINs that are localized in a deep region in the intermediolateral part of the gray matter. Further study is required to clarify the connectivity of the neuronal network within the upper cervical spinal cord.

Conclusion

To our knowledge, the work reported in the present paper is the first to provide evidence for spontaneous generation of respiratory rhythm in isolated upper cervical slice preparations. The present results suggest that a respiratory neuronal circuit, consisting of putative UCINs in the upper cervical (C1/C2) segments, under certain conditions has the potential for spontaneously generating a respiratory rhythm.

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