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Expressions of VGLUT1/2 in the inspiratory interneurons and GAD65/67 in the inspiratory Renshaw cells in the neonatal rat upper thoracic spinal cord

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ABSTRACT

Although the inspiratory spinal interneurons are thought to provide a major fraction of the excitatory synaptic potentials to the inspiratory intercostal motoneurons, this has not been confirmed. To clarify whether some inspiratory spinal interneurons are glutamatergic, we obtained whole-cell recordings from the ventromedial area of the third thoracic segments in an isolated brainstem-spinal cord preparation from neonatal rat, and the recorded cells were filled with Lucifer Yellow for later visualization. We then examined the existence of mRNA of vesicular glutamate transporters 1 and/or 2 (VGLUT1/2) by performing in situ hybridization. To discriminate the interneurons from motoneurons, we electrically stimulated the third thoracic ventral root on the recorded side, and the results verified that the antidromic spike or excitatory postsynaptic potential was not evoked. In cases in which the ventral root stimulation evoked depolarizing postsynaptic potentials, we examined the existence of glutamic acid decarboxylase 65 and/or 67 (GAD65/67) mRNA using a mixed probe to verify whether the cell was truly a Renshaw cell. The long diameter of the recorded interneurons was 22 ± 8 μm; the short diameter was 13 ± 4 μm. The interneurons' input resistance was 598 ± 274 MΩ. The Renshaw cells had similar sizes and input resistance. Six of 11 interneurons expressed VGLUT1/2, and four of five Renshaw cells expressed GAD65/67. Our findings suggest that approximately one-half of the inspiratory interneurons in the ventromedial area of the neonatal rat thoracic spinal cord are glutamatergic, and these interneurons might enhance the inspiratory intercostal motor activity.

Introduction

The basic respiratory rhythm and its motor patterns are generated by neuronal networks in the medulla that comprise the 'respiratory center' (Ezure, 1990; Bianchi et al., 1995; Onimaru et al., 1997). The respiratory center contains bulbospinal respiratory neurons as output neurons. These bulbospinal neurons regulate the motoneurons in the spinal cord that activate the various pump muscles, such as the diaphragm, external intercostal muscles, internal intercostal muscles, and abdominal muscles, in order to ventilate the lungs properly (Iizuka, 2011; Lane, 2011; Lee and Fuller, 2011). However, with the exception of the diaphragm (Lee and Fuller, 2011), it is largely unknown whether the bulbospinal respiratory neurons regulate the activity of the respiratory muscles monosynaptically or polysynaptically (Iizuka, 2011; Lane, 2011). For example, although bulbospinal inspiratory neurons are known to provide monosynaptic inputs to the intercostal inspiratory motoneurons (Davies et al., 1985a,b; Duffin and Lipski, 1987), these monosynaptic inputs were suggested to provide only a small fraction of the total depolarization needed for the discharge of the motoneurons (Davies et al., 1985b). In another study, the spike-trigger averaging of the membrane potential in an external intercostal motoneuron to the spikes of a bulbospinal inspiratory neuron was examined in 51 pairs, and monosynaptic connections between the pairs were not observed (Merrill and Lipski, 1987). Based on these results, Merrill and Lipski (1987) concluded that the bulbospinal inspiratory neurons regulate the external intercostal motoneurons via excitatory interneurons.

Extracellular and intracellular recordings have shown that many thoracic interneurons have respiratory activity (Kirkwood et al., 1988, 1993; Schmid et al., 1993; Saywell et al., 2011). The interneurons projecting to the thoracic ventral horn are distributed mainly in the contralateral medial ventral horn in the same spinal segment (Schmid et al., 1993). In their following study, they recorded from these respiratory interneurons, and described all five of the strongly modulated phasic inspiratory interneurons showed positive-going focal synaptic

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potential, indicating that these neurons are inhibitory (Kirkwood et al., 1993). Although the inspiratory spinal interneurons are thought to provide a major fraction of the excitatory synaptic potentials in the inspiratory intercostal motoneurons (Davies et al., 1985b; Merrill and Lipski, 1987), 'excitatory' inspiratory interneurons have not been found in the spinal cord.

The determination of the location of the excitatory inspiratory interneurons also has great importance when recovery from spinal cord injuries is considered. One of the primary causes of death among individuals who have suffered a spinal cord injury is pneumonia due to impaired respiratory function (National Spinal Cord Injury Statistical Center, 2016). It was demonstrated in a cat model that the hemisection of the spinal cord caused an immediate reduction in ipsilateral intercostal nerve activity below the injury site, but intercostal inspiratory activity recovered within a few days (Kirkwood et al., 1984), and in rats it returned within a few weeks (Dougherty et al., 2012; Zimmer et al., 2015). Based on these findings, it has been proposed that the intercostal muscles make a significant contribution to respiratory recovery after chronic cervical spinal cord injury (Dougherty et al., 2012), and plastic changes of the excitatory inspiratory interneurons in the spinal cord are suspected (Zimmer et al., 2015). Thus, the existence of the excitatory inspiratory interneurons in the spinal cord should be confirmed first.

It is well documented that the rostral part of the rib cage muscles of mammals shows larger inspiratory activity (De Troyer et al., 2005). Similarly, in an isolated brainstem spinal cord preparation from neonatal rat, the ratio of the thoracic inspiratory motor activity to the expiratory activity was larger in the rostral thoracic segment, suggesting that the neuronal mechanisms that generate the rostrocaudal gradient remain intact (Iizuka, 2004). A more recent study of a neonatal rat model showed that the inspiratory depolarizing optical signals in the motoneuron and interneuron area was larger in the more rostral thoracic spinal cord when the preparation was stained with a voltage-sensitive dye in the isolated brainstem-spinal cord preparation (Iizuka et al., 2016).

We hypothesized that some of the inspiratory interneurons in the rostral thoracic cord are excitatory and enhance the inspiratory motor outputs. Here we examined the existence of mRNA of vesicular glutamate transporters 1 and/or 2 (VGLUT1/2) by performing in situ hybridization with these inspiratory interneurons.

During the present experiment, some of the recorded cells showed excitatory postsynaptic potentials after electrical stimulation to the ventral root. Renshaw cells receive excitatory inputs from the motoneuron axon collaterals and exert a recurrent inhibition of synergist motoneurons (Alvarez and Fyffe, 2007). Although Renshaw cells are well-characterized inhibitory interneurons and have been studied especially in the lumbar spinal cord in relation to the regulation of locomotor activity (Nishimaru et al., 2006), the Renshaw cells and their location in the thoracic segments have not been fully examined (Kirkwood et al., 1981; Saywell et al., 2013). We therefore investigated the existence of mRNA of glutamic acid decarboxylase 65 and/or 67 (GAD65/67) on the recorded cells, which received depolarizing postsynaptic potentials evoked by ventral root stimulation, to verify whether these cells are truly Renshaw cells.

Methods

Ethical approval

This study was approved by the Animal Research Committee of Showa University, which operates in accordance with the Japanese Government's Law No. 105 for the care and use of laboratory animals.

Brainstem spinal cord preparation

Wistar rats (n = 23), 0–2 days of age, were deeply anesthetized with isoflurane until their nociceptive reflexes were abolished. The cerebrum was then quickly removed by transection at the intercollicular level, and the brainstem and spinal cord were isolated as described (Suzue, 1984; Onimaru et al., 1988). The brainstem was rostrally decerebrated between the 6th cranial nerve roots and the lower border of the trapezoid body. The spinal cord was cut at the level between the third and fourth thoracic (T3, T4) ventral roots.

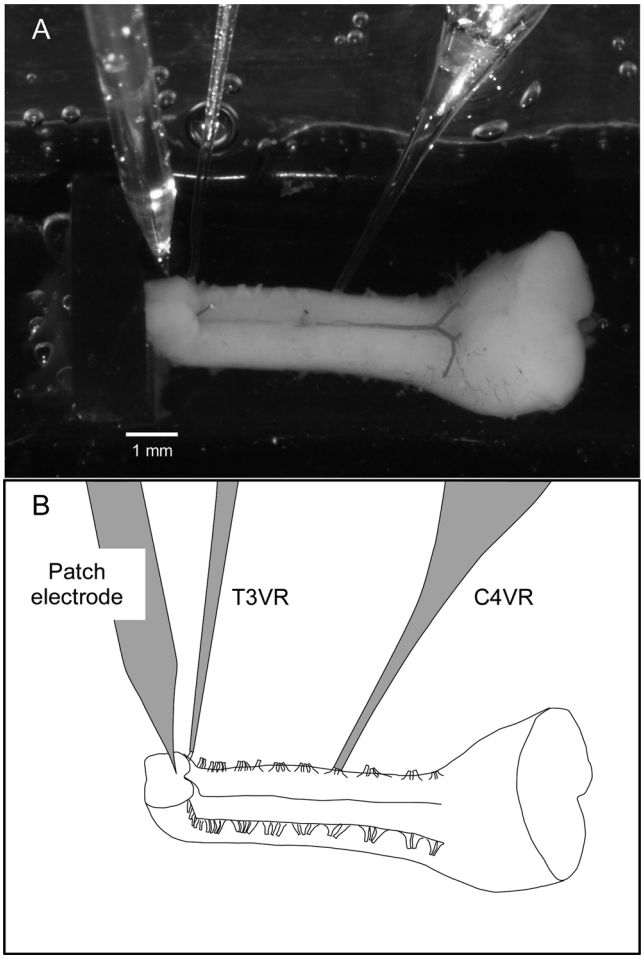

Fig. 1. Experimental arrangement. A: Photograph of a preparation. The preparation was placed with the ventral surface up and bent ventrally at around T1 to turn the section upward, and was pinned at the midline of C5 and T1 on an L-shaped silicone rubber plate (approximately 1-mm thick, 4–6 mm × 20–25 mm with a 2.5-mm-high vertical wall). The fourth cervical ventral root (C4VR) and the third thoracic ventral root (T3VR) were incorporated into glass suction electrodes. A glass electrode for the whole-cell patch-clamp recording was inserted from the surface of the cross-section. B: Schematic drawing of panel A.

As shown in Fig. 1, the brainstem-spinal cord preparation was placed with the ventral surface up and bent ventrally at around T1 to turn the section upward, and was pinned at the midline of C5 and T1 on an L-shaped silicone rubber plate. The preparation was then moved to a 2.5-ml perfusion chamber and superfused continuously at 2–3 ml/min with modified Krebs solution consisting of (in mM): 124 NaCl, 5.0 KCl, 1.2 KH_2PO_4, 2.4 $CaCl_2$, 1.3 $MgCl_2$, 26 $NaHCO_3$, 30 glucose, and equilibrated with 95% O_2 and 5% CO_2, pH 7.4, at 25°–27 °C.

Whole-cell patch-clamp recordings

The membrane potentials and input resistances of neurons in the ventromedial region of the section at the third thoracic spinal cord were recorded by a blind whole-cell patch-clamp method (Onimaru and Homma, 1992). The electrodes (inner tip diameter, 1.2–2.0 μm; resistance, 4–8 MΩ) were filled with the following pipette solution (in