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# Effects of riluzole on respiratory rhythm generation in the brainstem-spinal cord preparation from newborn rat

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ABSTRACT

The persistent sodium channel is an important pacemaker component in rhythm generation. In the present study, we examined the effects of a persistent sodium channel blocker, riluzole on pre-inspiratory (Pre-I) and inspiratory neurons in the rostral medulla as well as on 4th cervical ventral root (C4)-inspiratory activity in brainstem-spinal cord preparations. Preparations were isolated from postnatal day 0–3 Wistar rats and were superfused with artificial cerebrospinal fluid, equilibrated with 95% $O_2$ and 5% $CO_2$, pH 7.4, at 25–26 °C. The C4 inspiratory burst rate decreased in a dose-dependent manner (50–200 μM) after 15 min application of riluzole. Riluzole caused a strong reduction in the drive potential of Pre-I neurons but not of inspiratory neurons. After washout, C4 inspiratory burst gradually changed into an episodic pattern, in which one burst consisted of 3–9 short separate bursts. Riluzole also depressed the induction of repetitive firing induced by depolarizing stimulation. Under voltage clamp conditions, riluzole suppressed the negative-slope component of Pre-I neurons. Riluzole also depressed the intrinsic burst generation of Pre-I neurons in low calcium and high magnesium solution. Our findings indicate that the burst generation of Pre-I neurons is more sensitive than inspiratory burst generation to riluzole and thus suggested that persistent sodium channels have an important role in the burst generation of Pre-I neurons and are involved in the primary respiratory rhythm generation.



## 1. Introduction

In order to elucidate the mechanism by which respiratory rhythm is generated in the brainstem, it is necessary to clarify the contribution of the ionic currents. Persistent sodium current ($I_{NaP}$) is an important component of pacemaker properties in the rhythm generating neurons, including the respiratory center of the medulla (Ballanyi et al., 1999; Ramirez et al., 2011, 2004; Rybak et al., 2007). The persistent sodium channels are widely expressed in inspiratory (Insp) rhythm generating neurons of the pre-Bötzinger complex (preBötC) (Del Negro et al., 2002, 2005; Koizumi and Smith, 2008; Pace et al., 2007; Pena et al., 2004) and in several other types of bursting neurons (Brocard et al., 2006; Lamanauskas and Nistri, 2008; van Drongelen et al., 2006; Zeng et al., 2005). Riluzole, which is known to be a therapeutic agent for the treatment of amyotrophic lateral sclerosis (Gordon, 2013), has been used as a blocker of $I_{NaP}$ (Bellingham, 2011; Darbon et al., 2004; Del Negro et al., 2002; Ptak et al., 2005; Taccola and Nistri, 2007; Theiss et al., 2007; Urbani and Belluzzi, 2000). $I_{NaP}$ plays a major role in determining respiratory bursting patterns by amplifying synaptic excitatory inputs in preBötC neurons (Ramirez et al., 2004) and in intrinsic rhythm generation (Koizumi and Smith, 2008; Thoby-Brisson et al., 2009), although some studies have reported that it is unnecessary for respiratory rhythm generation *in vitro* (Del Negro et al., 2002; Pace et al., 2007).

It has been shown that pre-inspiratory (Pre-I) neurons in the parafacial respiratory group (pFRG) play an essential role in the generation of basic respiratory rhythm through functional interaction with preBötC Insp neurons (Lal et al., 2011; Onimaru et al., 2006, 2007). Although the role of $I_{NaP}$ in Pre-I burst generation of the pFRG has been suggested (Ballanyi et al., 1999; Thoby-Brisson et al., 2009), the detailed mechanism regarding its contribution to rhythm and burst generation remains to be analyzed. We hypothesized that $I_{NaP}$ could be one of the important currents in the burst generation of Pre-I neurons. With the exception of the report of Fong et al. (2009), there is little published research on the effects of riluzole on respiratory activity in the *in vitro* brainstem-spinal cord preparation that has been widely used in the field of respiratory control (Ballanyi et al., 1999). In the present study, we aimed to

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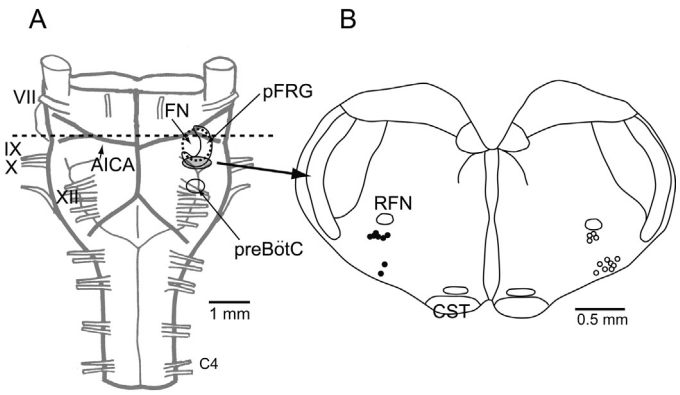


**Fig. 1.** The ventral view of a brainstem-spinal cord preparation from a newborn rat and the location of recorded neurons. (A) The ventral aspect. The preparations were cut transversely at a level just rostral to the anterior inferior cerebellar artery (dotted line). Neurons were recorded in the caudal part of the pFRG, denoted by a gray area. (B) Distribution of Lucifer-Yellow filled cells that were plotted in the corresponding slice where cells were recorded within ±100 μM of this slice level. Open circles, pre-inspiratory neurons; filled circles, inspiratory neurons; AICA, the anterior inferior cerebellar artery; C4, the fourth cervical ventral root; CST, corticospinal tract; FN, facial nucleus; pFRG, parafacial respiratory group; preBötC, pre-Bötzinger complex; RFN, retrofacial nucleus; VII–XII, cranial nerves.

examine the effects of riluzole on the burst generation of Pre-I neurons *in vitro* in brainstem-spinal cord preparations from newborn rats. This is the first report showing that riluzole causes a dose-dependent inhibition of Pre-I neuron burst generation in newborn rat preparations.

## 2. Materials and methods

### 2.1. Preparation and solutions

Brainstem-spinal cord preparations from postnatal day 0–3 Wistar rats were isolated under deep isoflurane anesthesia (Onimaru and Homma, 1992, 2003). The experimental protocols were approved by the Animal Research Committee of Showa University, which operates in accordance with Law No. 105 for the care and use of laboratory animals of the Japanese Government. The preparations were cut transversely at a level just rostral to the anterior inferior cerebellar artery (AICA) (Fig. 1A). Preparations were superfused continuously at 2.5–3 ml/min in a 2 ml chamber with artificial cerebro-spinal fluid (ACSF) (Suzue, 1984) composed 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, equilibrated with 95% $O_2$ and 5% $CO_2$; pH 7.4 and maintained at a temperature of 25–26 °C. Inspiratory activity corresponding to phrenic nerve activity was monitored from the fourth cervical ventral root (C4). Riluzole was purchased from Sigma–Aldrich (Tokyo, Japan) and was stocked as 100 mM solution in dimethyl sulfoxide. Drugs were dissolved with the above-described ACSF and bath-applied.

### 2.2. Whole-cell patch-clamp recording and histologic analysis

Membrane potentials of Pre-I and Insp neurons in the rostral ventrolateral medulla corresponding to the caudal part of the pFRG (Fig. 1) in which respiratory neurons have been recorded in a number of previous studies (Ballanyi et al., 2009; Onimaru et al., 2003, 2008; Onimaru and Homma, 2003) were recorded by a blind whole-cell patch-clamp method (Onimaru et al., 2003; Onimaru and Homma, 1992) with a high input impedance-DC amplifier (CEZ-3100; Nihon Koden, Tokyo, Japan). The electrodes, which had an inner tip diameter of 1.2–2.0 μm and a resistance of 4–8 MΩ, were filled with the following pipette solution (mM): 130 K-gluconate, 10 EGTA, 10 HEPES, 2 $Na_2$-ATP, 1 $CaCl_2$, and 1 $MgCl_2$, with pH 7.2–7.3 adjusted with KOH. We analyzed the membrane potential, input resistance, burst duration, and drive potential of Pre-I and Insp neurons (Ballanyi et al., 1999). The magnitude of the drive potential was determined as the voltage difference between the resting membrane potential in the interburst phase and the peak of the plateau depolarization during the burst phase.

Under the condition of blockade of potassium and calcium channels, detection of negative slope conductance is thought to be an indicator of the presence of $I_{NaP}$ (Crill, 1996; Del Negro et al., 2002; Koizumi and Smith, 2008; Li and Baccei, 2011; Moraes et al., 2013). In some experiments, we analyzed negative slope conductance in response to depolarizing voltage-ramp stimulation under voltage clamp conditions using a continuous voltage-clamp amplifier (current-voltage converter type) (Axopatch 1D, Axon Inc., Foster City, CA, USA). In the present study, we analyzed mainly Pre-I neurons, because many previous papers have already provided detailed analysis of Insp neurons (Koizumi et al., 2013; Koizumi and Smith, 2008). In this experiment, electrodes were filled with the following (potassium channel blockade) pipette solution (mM): 100 CsCl, 20 TEA-Cl, 11 K-BAPTA, 4 $Na_2$-ATP, 1 $CaCl_2$, 2 $MgCl_2$, 10 Hepes, and 0.5% Lucifer Yellow (lithium salt), with pH 7.2–7.3 adjusted with NaOH (Onimaru et al., 1996). After the establishment of whole cell recordings, we added 0.1 mM $CdCl_2$ into the external solution to block calcium channels. C4 activity disappeared within 10 min, and then the cell was clamped at −70 mV. To detect negative slope current, we tested slope of ramp stimulation in the range of 10–50 mV/s. Under our experimental conditions, contamination of fast sodium current was observed in most cases under all of the slope conditions tested, presumably due to an incomplete space clamp for the large dendritic field of these respiratory neurons (Ballanyi et al., 1999; Kawai et al., 2006; Onimaru and Homma, 1992). In most cases, we used 46.7 mV/s-ramp stimulation because the negative slope component was clearly detectable despite the contamination of the fast sodium current.

For histologic analysis of the recorded cells, the electrode tips were filled with 0.5% Lucifer Yellow (lithium salt). After experiments, preparations were fixed overnight at 4 °C in 4% paraformaldehyde in 0.1 M phosphate buffer solution (PBS), transferred into 18% sucrose/PBS and cut into 50 μm-thick transverse sections. Lucifer-Yellow-filled neurons were visualized using a fluorescence microscope (BX60, Olympus Optical, Tokyo, Japan), photographed and plotted in the corresponding slice. We confirmed that intracellularly recorded neurons were located in the caudal part of the pFRG (Fig. 1).

### 2.3. Synaptic transmission blockade solution

To investigate the direct effects of riluzole on intrinsic burst property of Pre-I neurons, we recorded extracellular unit activity using the same glass microelectrodes as those used for the above intracellular recordings. Then, chemical synaptic transmission was blocked by perfusing with low $Ca^{2+}$ (0.2 mM), high $Mg^{2+}$ (5 mM) solution (hereafter "low-Ca"; Onimaru et al., 1989) for more than 40 min. After disappearance of C4 inspiratory activity, the effects of riluzole on burst type Pre-I (Onimaru et al., 1989) were examined in low-Ca solution.

### 2.4. Data analysis

To assess the effects of riluzole on C4 or phrenic nerve activity, the burst rate (bursts/min) was calculated from the mean rate for 3–5 min. The duration of C4 activity was averaged from 10 consecutive respiratory cycles. Data are presented as mean and standard deviation (SD) for all preparations. A paired *t*-test was used to compare the inspiratory burst rate, amplitude and burst duration before