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A novel statistical analysis of voltage-imaging data by structural time series modeling and its application to the respiratory neuronal network

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ABSTRACT

The respiratory neuronal network activity can be optically recorded from the ventral medulla of the *in vitro* brainstem–spinal cord preparation using a voltage-sensitive dye. To assess the synchronicity between respiratory-related neurons and the breath-by-breath variability of respiratory neuronal activity from optical signals, we developed a novel method by which we are able to analyze respiratory-related optical signals without cycle-triggered averaging. The model, called the sigmoid and transfer function model, assumes a respiratory motor activity as the output and optical signals of each pixel as the input, and activity patterns of respiratory-related regions are characterized by estimated model parameter values. We found that rats intermittently showing multi-peaked respiratory motor activities had a relatively low appearance frequency of respiratory-related pixels. Further, correlations between respiratory-related pixels in rats with such unstable respiratory motor activities were poor. The poor correlations were caused by respiratory neurons recruited in the late inspiratory phase. These results suggest that poor synchronicity between respiratory neurons, which are recruited at various timings of inspiration, causes intermittent multi-peaked respiratory motor output. In conclusion, analyses of respiratory-related optical signals without cycle-triggered averaging are feasible by using the proposed method. This approach can be widely applied to the analysis of event-related optical signals.

1. Introduction

Neuronal network that generates respiratory rhythm and pattern is located mainly in the lower brainstem (Feldman et al., 2003; Ezure, 2004; Feldman and Del Negro, 2006). The respiratory network activity can be optically recorded from the ventral medulla *in vitro* using a voltage-sensitive dye (Onimaru and Homma, 2003; Okada et al., 2007a,b; Oku et al., 2007, 2008a,b). We have previously shown that correlation coefficient imaging, where the maximal correlation coefficient between a respiratory motor nerve output signal and a time-lagged optical signal of each pixel is plotted on a reference image, is a powerful tool to map respiratory-related regions (Okada et al., 2007b; Oku et al., 2007, 2008a,b). Using the lag in which the cross-correlation becomes the maximum, the method could differentiate the dynamic characteristics between the two respiratory-related regions, the parafacial respiratory group (pFRG)/retrotrapezoid nucleus (RTN) (Onimaru and Homma, 2003) region and the preBötzinger complex (preBötC)/ventral respiratory group (VRG) region (Smith et al., 1991). However, since this method requires a cycle-triggered averaging to improve the signal-to-noise ratio, the interrelationship between respiratory-related areas or the breath-by-breath variation of neuronal activity could not be assessed. Therefore, we have recently developed a new method that enables to analyze the respiratory neuronal activity without averaging signals (Kawai et al., in press). The new method adopts a parametric model, called the sigmoid and transfer function (STF) model. The STF model assumes respiratory motor nerve activity as the output and optical signals of each pixel as the input, and the dynamics of respiratory-related regions are characterized by estimated model parameter values.

In the present study, to get insights into the mechanisms of the respiratory rhythm and pattern generation, we applied the STF model to experimental data obtained from *in vitro* preparations

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and evaluated the synchronicity between respiratory-related pixels within a breath and the breath-by-breath variation of respiratory neuronal activities. We found that the synchronicity between respiratory-related pixels dramatically changes coincident with the stability of the respiratory motor output. Further, by introducing a new functional image, i.e., frequency distribution image, we found that the same activity pattern does not always appear on each pixel at every recording. The implications of this result are discussed.

2. Methods

2.1. Animal preparation

Isolated brainstem–spinal cord preparations were made of neonatal Sprague–Dawley rats (n = 10, 0–4 days old) as described previously (Suzue, 1984; Okada et al., 2007b; Oku et al., 2007, 2008b). Experimental protocols were approved by the Animal Research Committee of Hyogo College of Medicine. Briefly, each animal was deeply anaesthetized with diethyl ether, quickly decerebrated at the intercollicular level, and the brainstem and spinal cord were isolated. The recording chamber was continuously superfused with an artificial cerebrospinal fluid (aCSF) at a rate of 3 ml/min. The aCSF contained (mM): NaCl 124, KCl 5.0, $CaCl_2$ 2.4, $MgSO_4$ 1.3, KH_2PO_4 1.2, $NaHCO_3$ 26, glucose 30, and was equilibrated with 5% CO_2 and 95% O_2. The pH was 7.4 at the experimental condition. The temperature of the superfusate was controlled at 27 ± 1 °C. Inspiratory-related neural activity was monitored from the C4 ventral root (C4VR) with a suction electrode and was used as respiratory motor output. The raw nerve signal was amplified and band-pass filtered from 15 Hz to 3 kHz. The filtered signal was used to trigger an optical recording system.

2.2. Optical recording

The preparation was incubated in an aCSF containing a voltage-sensitive dye, di-2-ANEPEQ (0.1–0.2 mM, Invitrogen, Carlsbad, California, USA) for 40 min with 95% O_2 and 5% CO_2 (Okada et al., 2007b; Oku et al., 2007, 2008b). Activity of respiratory neurons in the ventral medulla was analyzed using a fluorescence macro-zoom microscope (MVX-10, Olympus Optical, Tokyo, Japan) and an optical recording system (MiCAM Ultima, BrainVision, Tokyo, Japan). The focal plane was adjusted at 100–200 μm deep from the surface of the ventrolateral medulla at the region that we assumed to be the preBötC/VRG by monitoring respiratory-related optical signals. Preparations were illuminated through a band-pass excitation filter (λ = 480–550 nm), and epifluorescence through a long-pass barrier filter (λ > 590 nm) was detected with a CMOS sensor array. Magnification of the microscope was adjusted to 2.8× to 3.3× depending on the size of each preparation. One pixel corresponded to 30 × 30 to 35 × 35 μm, and the image sensor covered a total of 3 × 3 to 3.5 × 3.5 mm^2. Optical signals were sampled at 50 Hz. A total of 256 frames were recorded, and the first 64 frames were taken before the onset of inspiratory C4VR activity.

2.3. Data analysis

To analyze respiratory-related neuronal activity without cycle-triggered averaging, we preprocessed the C4VR respiratory motor output and optical signals as follows. After removing the linear trend, the C4VR respiratory motor output was normalized using the maximum ($C4VR_{max}$) and the minimum ($C4VR_{min}$) values as follows:

$$\text{Normalized C4VR} = \frac{\text{Measured C4VR}}{C4VR_{max} - C4VR_{min}} \quad (1)$$

After removing linear trends, optical signals were moving-time averaged (bin width = 7) and spatially averaged by 3 × 3 pixels to remove high frequency noises. Subsequently, the STF model was applied to the preprocessed optical signals as follows. Suppose that $x(s)$ is an optical time series datum of a given pixel, and $y(s)$ is the C4VR respiratory motor output. Then $y^*(s)$, the estimate of $y(s)$, is formulated by the STF model (Kawai et al., in press):

$$y^*(s) = \frac{Ke^{-Ls}}{1+Ts} \times \frac{1}{1+e^{-(x(s)-a)}} \quad (2)$$

where a, K, L and T represent threshold, gain, dead time (delay) and time constant, respectively. The STF model is composed of a sigmoid function for thresholding the input (the second term) and a transfer function for accounting the delay (the first term), after which the model was named. The parameter values were determined so that the variance of estimation error was minimized. Given that the sampling interval is Δt, the dead time L is expressed as $L = l\Delta t + \Delta l$, where l is an integer. Eq. (2) is rewritten in a discrete form:

$$y^*(n) = 2d \times y^*(n-1) - d^2 y^*(n-2) + (1 - d)\frac{K \times d^m}{1+e^{-(x(n-l-1)-a)}} \quad (3)$$

$$\text{where } d = e^{\Delta t/T},\ m = \frac{\Delta l}{\Delta t}$$

The variance of estimation error $\sigma^2(e)$ is expressed as

$$\sigma^2(e) = \frac{1}{N}\sum_{n=1}^{N}(y(n) - y^*(n))^2 \quad (4)$$

Then, a, T, K and L that minimize $\sigma^2(e)$ were estimated by one of non-linear optimization methods, the sequential quadratic programming method (Gill et al., 1981, 1984).

To detect and classify respiratory-related pixels, we introduced the estimation error ratio, which is defined as $R = \sigma(e)/\sigma(y)$. Respiratory-related pixels were defined as pixels having estimated parameter values and estimation error ratio within certain ranges. In the previous studies (Kawai et al., in press), we applied the STF model to 17 breaths of a post-natal day 0 (P0) rat brainstem spinal cord preparation. The STF model was applied to preprocessed time series data of all (100 × 100) pixels, and model parameter values were estimated for each pixel of each breath. We then mapped the means and the standard deviations of estimated STF parameter values and estimation error ratio of each pixel on the reference image. We found that respiratory-related pixels could be reasonably classified into five categories by dead time, gain, and estimation error ratio. The spatial distribution of classified respiratory-related pixels was consistent with that obtained by the correlation coefficient mapping using cycle-triggered averaging data (Oku et al., 2007), and characterized the activity pattern of respiratory-related pixels more in detail. In the present study, we took the advantage of this method, and analyzed optical signals obtained from 10 rats. STF model parameters and estimation error ratios were calculated for each pixel of each breath using preprocessed time series data. The criteria of the classification were refined from the physiological viewpoint and shown in Table 1. Each activity type has the following characteristics:

- Type-1 has a relatively small estimation error ratio and a positive large dead time.
- Type-2 has a small estimation error ratio and a positive small dead time or a negative small dead time.
- Type-3 has a small estimation error ratio and a negative large dead time.
- Type-4 has a large estimation error ratio and a negative large dead time.