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Respiratory calcium fluctuations in low-frequency oscillating astrocytes in the pre-Bötzinger complex☆

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ABSTRACT

Astrocytes have been found to modulate neuronal activity through calcium-dependent signaling in various brain regions. However, whether astrocytes of the pre-Bötzinger complex (preBötC) exhibit respiratory rhythmic fluctuations is still controversial. Here we evaluated calcium-imaging experiments within preBötC in rhythmically active medullary slices from TgN(hGFAP-EGFP) mice using advanced analyses. 13.8% of EGFP-negative cells, putative neurons, showed rhythmic fluorescent changes that were highly correlated to the respiratory rhythmic fluctuation (cross-correlation coefficient > 0.5 and $dF/F > 0.2\%$). In contrast, a considerable number of astrocyte somata exhibited synchronized low-frequency (<0.03 Hz) calcium oscillations. After band-pass filtering, signals that irregularly preceded the calcium signal of EGFP-negative cells were observed in 10.2% of astrocytes, indicating a functional coupling between astrocytes and neurons in preBötC. A model simulation confirmed that such preinspiratory astrocytic signals can arise from coupled neuronal and astrocytic oscillators, supporting a concept that slow oscillatory changes of astrocytic functions modulate neighboring neuronal activity to add variability in respiratory rhythm.

1. Introduction

Astrocytes have been recognized to play active roles in various brain regions through bidirectional communication with neuronal circuits (Fellin, 2009), and are able to facilitate neuronal synchronization (Angulo et al., 2004; Tian et al., 2005). In the respiratory network, astrocytes have been shown to be vital for ongoing neuronal activity (Hulsmann et al., 2000) and they were postulated to play a crucial role in central chemoreception (Gourine et al., 2010). Astrocytes often show intrinsic calcium oscillations independent of neuronal activity that propagate to neighboring astrocytes and modulate neuronal activity by releasing gliotransmitters (Parri et al., 2001; Pasti et al., 1997; Scemes and Giaume, 2006) or regulating extracellular neurotransmitter concentration (Gomeza et al., 2003; Schnell et al., 2011). Slow intrinsic calcium oscillations have also been found in the pre-Bötzinger complex (preBötC) of mice using slice preparations (Hartel et al., 2009; Schnell et al., 2011). However, the roles these astroglial calcium oscillations play in the respiratory network have not been elucidated yet.

Recently, controversial data have been published for the preBötC network regarding the existence of calcium oscillations that are coupled to the neuronal activity. While the group of one of the authors of this paper (S.H.) reported respiratory rhythmic membrane currents in astrocytes of the neonatal mouse preBötC, without finding evidence for respiratory rhythmic calcium signals, Okada and Colleagues together with Y.O. shortly thereafter reported preinspiratory calcium signals in preBötC of rat (Okada et al., 2012; Schnell et al., 2011). In the present study, we therefore reevaluate calcium imaging experiments within the preBötC that have been conducted with a multifocal multi-photon microscope using Oregon green BAPTA-1 AM (OGB-1) as a calcium indicator, and transgenic TgN(hGFAP-EGFP) mice that express the green fluorescent protein in astrocytes, using rhythmically active medullary slices (Schnell et al., 2011). Although the data have been analyzed before (Schnell et al., 2011), different analysis methods were now

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used to allow automatized detection of time-lagged calcium signals that are correlated with the neuronal respiratory network activity. Interestingly, we could identify not only low frequency oscillation as described before (Hartel et al., 2009; Schnell et al., 2011), but also some irregularly occurring calcium fluctuations preceding neuronal activity in 10% of preBötC astrocyte. In order to gain insights in the potential physiological significances of such pre-inspiratory astrocytic calcium activity, we simulate the behavior of weakly coupled neuronal and astrocytic oscillators using a mathematical model.

2. Methods

For this paper we used a dataset from hGFAP-EGFP transgenic mice that was collected for a previously published study (Schnell et al., 2011). Since analysis algorithms have been advanced since the publication has been submitted, and since there was a paper published showing opposing data from rats (Okada et al., 2012), we decided to reevaluate the mouse data. Methods different from an earlier publication (Schnell et al., 2011) are described in Sections 2.3–2.6.

2.1. Animal handling

Animals were bred in the animal facility of the University Hospital Göttingen and treated in accordance with the guidelines of the German Physiological Society as well as the regulations of the State of Lower Saxony and the Federal Republic of Germany. The institutional permit number was T19.08. Neonatal mice (TgN(hGFAP-EGFP)$^{GFEC\text{-}Fki}$; postnatal day 0–11) that have astrocytes in the respiratory network labeled with the enhanced green fluorescent protein (Grass et al., 2004; Nolte et al., 2001) were used.

2.2. Two-photon calcium imaging data collection

Methods of respiratory-related rhythm generating medullary slice preparation and multi-cell bolus loading into the preBötC were as described previously (Schnell et al., 2011). Calcium imaging was performed using a two-photon laser-scanning system (TriMScope, LaVision BioTec, Bielefeld, Germany) with a 40× (0.8 NA) water-immersion objective lens (Zeiss; Oberkochen, Germany) (Winter et al., 2009). OGB-1 fluorescence was detected at 800 nm excitation wavelength through a YFP-filter (BP 511–551 nm), whereas EGFP-fluorescence was detected through a CFP-filter (475–500 nm) with 900 nm excitation wavelength (Winter et al., 2009). Images were taken using a CCD-camera (Ixon 885; Andor Technology, Belfast, Northern Ireland, or PCO; Sensicam QE; Kehlheim, Germany) at 0.1 s/frame, and exported to TIFF format for later analysis using Matlab (Mathworks Inc., Natick, MA, USA).

2.3. Data processing and automatic cell detection

Data were band-pass filtered (cutoff frequencies: 0.05–1 Hz), and converted into dF/F% by dividing change of fluorescence intensity of each pixel (dF) by the average fluorescence intensity of the initial 10 frames of each pixel (F). To generate overlay images that allow later analyses, i.e. localization and discrimination of putative neurons (EGFP-negative) and astrocytes that exhibit calcium fluctuations related to respiratory rhythm, we overlaid EGFP signal, OGB-1 fluorescence, and cross-correlation image (Oku et al., 2007). The cross-correlation image (CCI) was made using the average calcium signal of the entire frame as the reference function, then spatially filtered (5 × 5 pixels). This spatially filtered CCI was pseudo-color mapped in red to indicate cells with respiratory-correlated fluctuation. The spatially filtered (5 × 5 pixels) EGFP signal was pseudo-color mapped in green to indicate GFAP-positive astrocytes. The raw OGB-1 fluorescence, averaged for the first 10 frames and spatially filtered (5 × 5 pixels), was pseudo-color mapped in blue to indicate locations of both neurons and astrocytes that were labeled with OGB-1. Finally these three images were overlaid (see Fig. 3B).

The locations of astrocytes in a frame were automatically identified as follows. First, local maxima, whose EGFP fluorescence intensity was greater than the mean of the entire frame, were searched within the scope of 15 × 15 pixels. Then temporal changes of mean OGB-1 fluorescence of 7 × 7 pixels ROIs centered on the local EGFP-fluorescent maximum were calculated to obtain calcium transients of each astrocyte (EGFP-positive).

Both OGB-1 signals and absence of EGFP fluorescence were used to automatically identify locations of EGFP-negative cells (putative neurons). First, local maxima of OGB-1 fluorescence, whose intensity were greater than the mean of the entire frame, and EGFP fluorescence intensity was less than its mean of the entire frame were searched within the scope of 15 × 15 pixels. Then, temporal changes of mean OGB-1 fluorescence of 7 × 7 pixels ROIs centered on the local maximum were calculated to obtain calcium transients of each EGFP-negative cells.

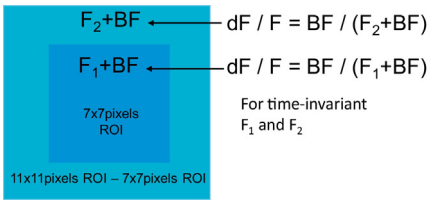

Fig. 1. Measurement of background fluorescence. F_1 and F_2 represent intensities of inherent calcium fluorescence of 7 × 7 pixels ROI and its adjacent pixels, respectively. BF denotes background fluorescence. If F_1 and F_2 were time-invariant and did not show respiratory-related calcium fluctuations, dF/F of the cell and its vicinity are BF/(F_1 + BF) and BF/(F_2 + BF), respectively. See text.

2.4. Detection of low frequency oscillations in EGFP positive cells

To detect low frequency oscillations in EGFP positive cells, autocorrelation function was calculated with lags ranging between −100 and 100 s after low-pass filtering (cutoff frequency: 1 Hz) for each EGFP positive cell whose fluctuation amplitude is greater than 0.4 dF/F%, which is about 10 times the baseline noise. Then the autocorrelation function was smoothed by applying moving time averaging (window width 5 s) twice, and peaks were searched at the frequency range between 0.015 and 0.05 Hz. If multiple peaks were detected, then the highest peak was chosen for oscillation frequency of the cell.

2.5. Estimation of out of focus light artifacts

The original paper used a rolling ball algorithm from ImageJ to eliminate background fluorescence (Schnell et al., 2011). Here we compared the analysis with and without background subtraction, but additionally evaluated the influence of out of focus fluorescence artifacts originating from cellular compartments below or above the focal cell by determining the OGB-1 calcium signal in the soma as well as in the vicinity of the cell. Therefore, we set an 11 × 11 pixel ROI centered on the soma, and calculated the average calcium signal inside and four quadrants of outside the 7 × 7 pixels ROI (Fig. 1). The cellular calcium signal observed in the 7 × 7 pixels is the inherent cellular calcium signal (F_1) plus out of focus signal (BF_0), and the calcium signal observed outside the 7 × 7 pixels ROI is the signal emitted from the focal plane (F_2) plus BF_0.