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ULTRA-SLOW OSCILLATIONS IN CORTICAL NETWORKS *IN VITRO*

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Abstract—An ultra-slow oscillation (<0.01 Hz) in the network-wide activity of dissociated cortical networks is described in this article. This slow rhythm is characterized by the recurrence of clusters of large synchronized bursts of activity lasting approximately 1–3 min, separated by an almost equivalent interval of relatively smaller bursts. Such rhythmic activity was detected in cultures starting from the fourth week *in vitro*. Our analysis revealed that the propagation motifs of constituent bursts were strongly conserved across multiple oscillation cycles, and these motifs were more consistent at the electrode level compared with the neuronal level.

Key words: ultra-slow oscillation, cortical culture, multi-electrode array, low-frequency stimulation, repeating motif.

Large-scale rhythmic oscillation is the hallmark of brain physiology and is believed to underlie diverse processes of the nervous system. The frequency of electroencephalographic oscillation encompasses a multitude of frequency bands that varied with the functional state of the brain. Slow rhythm (<4 Hz) is particularly crucial in deep sleep stages, where it has been detected over widespread regions in the thalamocortical networks. The broad range of the slow rhythm belongs to at least three different types of oscillatory activities with each playing potentially different roles, including delta rhythm ranging from 1 to 4 Hz (Steriade et al., 1993a,b), slow oscillation with frequency <1 Hz (Metherate and Ashe, 1993; Steriade et al., 1993a,b; Contreras et al., 1996; Achermann and Borbély, 1997), and ultra-slow oscillatory activity that recurred on the order of 0.001–1 Hz (Penttonen et al., 1999; Vanhatalo et al., 2004; Drew et al., 2008).

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Abbreviations: FFT, fast Fourier transform; IBI, inter-burst interval; ISI, inter-spike interval; MEA, multi-electrode array.

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Although the slow rhythm was once thought to arise only within local networks *in vivo*, there is compelling evidence that slow network oscillations could be expressed *in vitro* (Timofeev et al., 2000). The study of this recurrent activity in *in vitro* networks is potentially useful, as it leads to a better understanding of the slow rhythm in intact brains. Previous experiments with cortico-thalamic slices have provided important insights on the cellular and network mechanisms that may account for the generation of slow rhythm (Sanchez-Vives and McCormick, 2000; Hughes et al., 2002; Shu et al., 2003; Lõrincz et al., 2009), as well as the effect of varying the temperature of bath solution on cortical rhythms (Reig et al., 2010). However, slow network oscillations on the time scale of minutes have not been shown in dissociated cortical culture that is lacking of the specific cytoarchitecture *in vivo*.

Here, we demonstrate the spontaneous emergence of a very slow network oscillation (<0.01 Hz), hereafter termed ultra-slow oscillation, in dissociated cortical networks in culture. Our results indicate that the propensity of this rhythmic activity was enhanced with the application of low-frequency electrical stimulation. We also found repeating motifs of bursts in the ultra-slow oscillations, based on the analysis of their propagation profiles during burst initiation.

EXPERIMENTAL PROCEDURES

Cell culture

Embryos were obtained from timed-pregnant Wistar rats euthanized using CO_2 at day 18 of gestation. The cortices were dissected and cut into small pieces in ice-cold Hanks' balanced salt solution (Invitrogen, Carlsbad, CA, USA). This is followed by enzymatic digestion by incubation in 0.25% trypsin (Invitrogen) at 37 °C for 15 min. The partially digested tissues were mechanically triturated using 1-ml pipette tips and centrifuged at 150×*g* for 6 min. The cells were fully dissociated by gentle trituration and diluted into 2500 cells/μl in Neurobasal medium (Invitrogen) supplemented with 5% equine serum (Hyclone, Logan, UT, USA), 2% B27 (Invitrogen), and 0.05 mM Glutamax (Invitrogen). Before plating, we mixed the cell suspension with 0.01 mg/ml laminin (Sigma, St. Louis, MO, USA). Subsequently, 50 μl of cells were deposited onto the central region of each multi-electrode array (MEA), which was pre-coated with poly-ethylene-imine (Sigma). The cells were allowed to attach onto the substrates for 2 h, before flooded with 1 ml of medium. The cultures were sealed with Teflon membranes (Potter and DeMarse, 2001) and kept inside the incubator. Half of the medium was replaced every 4–6 days.

Electrical recording and stimulation

Cortical neurons were cultured on MEAs (MultiChannel Systems, Reutlingen, Germany) with 60 titanium nitride electrodes, 30 μm in diameter, laid out on an 8x8 grid with 200 μm inter-electrode spacing. Electrical activity from the cultures was amplified 1100×

using a commercial 60-channel amplifier (MEA1060-Inv-BC, MultiChannel Systems) with frequency limits of 10 Hz to 3 kHz. Signals were sampled at 25 kHz at 14-bit resolution using a data acquisition card (MC Card, MultiChannel Systems). Online visualization was performed with MC Rack software (MultiChannel Systems), whereas spike detection and the subsequent data analysis were performed using Matlab (MathWorks).

Cultures were probed with biphasic voltage pulses of ±800 mV, 400 μs per phase (positive phase first) twice a week starting from the first week *in vitro* using a custom-made stimulator. The stimuli were applied sequentially at 3-s intervals through eight electrodes in the fifth column of MEAs, for a total duration of 3.5 h. In all experiments, spontaneous activity was recorded immediately before and after the stimulation. For the purpose of comparison with non-chronically stimulated (control) cultures, we also monitored the activity of the latter group before and after the application of the same stimulus. Experiments on the control group were performed using new culture each time, to prevent any unintentional effect which might be caused by earlier stimulation.

All experiments were performed inside the same incubator used for maintaining cultures. To stabilize the temperature of the MEA substrates, excess heat from the amplifier was dissipated with our custom-made heat exchanger. Experiments were started 30–40 min after transferring the cultures to the amplifier. We scheduled all our experiments at least 12 h after feeding to avoid electrophysiological transients (Potter and DeMarse, 2001; Wagenaar et al., 2006a).

Data analysis

Spike detection and sorting. The voltage traces were first high-pass filtered at 200 Hz to remove the low-frequency postsynaptic potential components, yielding a flat baseline. Spikes were then detected by thresholding at 7.5× estimated standard deviation of the background noise (Quiroga et al., 2004). Subsequently, candidate spikes of smaller amplitude within a ±1-ms window were discarded, to prevent duplicate detection of multi-phasic spikes (Wagenaar et al., 2005).

Single-unit activity was isolated by spike sorting. We used a modified version of WaveClus (Quiroga et al., 2004), in which we applied the discrete derivative method for feature extraction (Nadasdy et al., 2002; Gibson et al., 2010) and superparamagnetic clustering for clustering the feature space. Up to four single units were isolated from each electrode. Single units were validated by a 3-ms refractoriness test. Spike times were resampled at 1 ms.

Burst detection. Bursts were identified according to a method previously described (Wagenaar et al., 2005). We first extracted sequences of at least four densely spaced spikes with a maximum inter-spike interval (ISI) of 100 ms as the core of bursts on individual electrodes. They were later extended to include entourage of spikes on the same electrodes with ISIs less than 200 ms. A network burst is then defined as an episode of temporally overlapping burst activities spanning five or more electrodes over the entire array.

Periodicity. To better examine and illustrate the periodicity of the network activity, fast Fourier transform (FFT) analysis was performed on population firing rate obtained with a bin width of 4 ms. Before FFT, the DC component was removed by subtracting the mean from the data. The analysis often yielded more than one dominant frequency over the spectrum of interest, as the intervals between successive bursts varied slightly from one another. For the subsequent analysis, the oscillation frequency is defined as the highest peak within the frequency band of 0–0.01 Hz.

Similarity indices. A burst similarity index (S_{burst}) was computed based on the method described in Raichman and Ben-Jacob (2008). For each burst, we searched for the first spikes on all the active electrodes. The latency between electrode pair (*i, j*) during the initiation of burst *n*, $t_n(i, j)$, was obtained by calculating the relative difference in the timings of their first spikes. The similarity index between burst *n* and *m* was then defined as the fraction of electrode pairs with the difference in their latencies between the two bursts less than a prespecified threshold, t_c:

$$S_{burst}(n,m) = \frac{1}{N(N-1)} \sum_{i \neq j} H(t_c - |t_n(i,j) - t_m(i,j)|)$$

where H is the Heaviside-step function and N is the number of active electrodes.

The calculation was repeated using neuron pairs (*i, j*) obtained from spike sorting. Only neurons with mean firing rate of at least 0.5 spikes per second were included in the analysis. We set $t_c = 0.03$ (Raichman and Ben-Jacob, 2008) in both analysis at the electrode and neuronal level.

RESULTS

Extracellular recordings revealed the spontaneous emergence of ultra-slow oscillations in the network-wide aggregate firing rates of chronically stimulated and control cultures during the fourth to sixth week *in vitro*. In both groups of cultures, an increased propensity of such rhythmic patterns was detected in the network activity after probing the cultures with low-frequency electrical pulses (see Experimental Procedures). This slow rhythm was detected in four recordings before stimulation and in eight recordings after stimulation out of the total 20 recordings made from chronically stimulated cultures ($n=5$ cultures). In control cultures, the rhythmic activity was exhibited in three and five separate cultures out of 13 cultures before and after stimulus delivery, respectively. This slow periodicity persisted with high fidelity over hours once it has developed in the culture, regardless of whether the latter was exposed to chronic stimulation.

The ultra-slow oscillations were shown as a succession of up and down states at frequencies of 0.002–0.008 Hz. The aggregate firing rates at peaks were on average 2–4 times higher than at troughs, with the majority of the spikes contained in bursts of different sizes (Fig. 1A). The oscillation frequency of such sequences was manifested as a prominent peak in the power spectral density (Fig. 1B). Bursts were separated by intervals of 2–5 s, with longer inter-burst intervals (IBIs) preceded bursts with higher number of spikes (Fig. 1C), implying that burst sizes were determined by parameters recovered from previous bursts. At neuronal level, the total number of cells contributing to a burst steadily increased with the increase of burst sizes toward the peaks, then leveled off. The average number of spikes per neuron also similarly varied when transitioning from the troughs to the peaks of the oscillations (Fig. 1D).

To investigate whether the spatiotemporal dynamics of activity was conserved across the constituent bursts, an analysis was performed to quantify the similarity in their propagation profiles based on the latencies between individual electrodes during burst initiation (see Experimental Procedures). The similarity index was found to be especially high between bursts at peaks, but was on average much lower between constituent bursts