Asynchronously Enhanced Spiking Activity of Ischemic Neuronal Networks

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Received January 20, 2011; accepted January 29, 2011; published online February 9, 2011

Cerebral ischemia causes the depletion of oxygen and nutrition from brain tissues, and when persistent, results in irreversible damage to the cell function and survival. The cellular response to ischemic conditions and its mechanisms have been investigated widely in *in vivo* and *in vitro* experimental models, yet no study has addressed the response of a whole neuronal network to energy deprivation with the single-cell resolution. Observations at the level of network are necessary, because the activity of individual neurons is nonlinearly integrated through a network and thereby gives rise to unexpectedly complex dynamics. Here we used functional multineuron calcium imaging (fMCI), an optical recording technique with high temporal and spatial resolution, to visualize the activity of neuron populations in hippocampus CA1 region under ischemia-like conditions *ex vivo*. We found that, although neurons responded to oxygen and glucose deprivation with an increase in the event frequency, they maintained an asynchronous network state. This is in contrast with other well known pathological states, in which the network hyperexcitability is usually accompanied by an increase in synchrony. We suggest that under ischemic conditions, at least to some time point, the neuronal network maintains the excitatory and inhibitory balance as a whole, whether actively or as a consequence of the cellular response to energy deprivation.

Key words asynchrony; ischemia; calcium imaging; neuronal network

Cerebral ischemia drawn by stroke, cardiac arrest, and other circulatory disorders induces complex neuronal responses leading to irreversible dysfunction and cell death. The mechanisms of these responses and their consequences have been investigated widely both *in vivo* and *in vitro*, including changes in neurotransmitter release, uptake, and degradation,[1—5] receptor alteration,[1,6,7] enhancement of proteolysis,[8,9] and mitochondrial metabolic aberration.[9,10] But these studies are conducted only at a single-cell level, and how the whole neuronal network reacts to energy deprivation has not been assessed. This point of view is important, because the activity of each cell is summed up in a network, producing complex responses to environment changes.

Here we simulated *ex vivo* ischemia by applying oxygen-glucose deprivation (OGD) to rat hippocampal organotypic slice cultures and captured the activity of an assembly of CA1 neurons using functional multineuron calcium imaging (fMCI), an optical technique that records suprathreshold activities *en masse* from large neuron populations with single cell resolution. We discovered that the network shows enhanced neuronal activity but maintains the same asynchrony level as control, as confirmed by two mathematically irrelevant parameters. This is distinct from other well known pathological states in which the network excitability rises with synchrony. This previously unknown network alteration brought by energy deprivation should open a novel strategy toward therapeutic and pharmaceutical approach to ischemia.

MATERIALS AND METHODS

Organotypic Hippocampal Slice Cultures and Recording Solutions All experiments were performed with the approval of the animal experiment ethics committee at the University of Tokyo according to the University of Tokyo guidelines for the care and use of laboratory animals. Hippocampal slices (300 μm) were prepared from postnatal day 7 Wistar/ST rats (SLC, Shizuoka, Japan), embedded on an Omnipore membrane filter with culture media, and incubated in 5% CO_2 at 35 °C.[11] The slice cultures used in the experiments were maintained 7 to 14 d *in vitro* after preparation. For the experiments, slice cultures were transferred to a heated recording chamber (29—33 °C) and continuously perfused with carbonated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (aCSF) containing 126 mM NaCl, 26 mM $NaHCO_3$, 3.5 mM KCl, 1.25 mM NaH_2PO_4, 1.3 mM $MgSO_4$, 2.0 mM $CaCl_2$, and 10 mM glucose. For OGD, the extracellular solution was switched to aCSF without glucose, bubbled with 95% N_2–5% CO_2.

Electrophysiology Cell-attached recordings were taken from the soma of CA1 cells with Axopatch 700B amplifier (Molecular Devices, Union City, CA, U.S.A.). Patch pipettes (4—9 MΩ) were filled with normal aCSF.

Functional Multineuron Calcium Imaging (fMCI) Slices were incubated for 45 min at 35 °C with 0.0005% Oregon Green 488 BAPTA 1-AM (Invitrogen), 0.01% Pluronic F-127 (Invitrogen), and 0.005% Cremophor EL (Sigma-Aldrich, St. Louis, MO, U.S.A.) and then recovered in aCSF. Images were collected at 10 frames/s with a Nipkow-disk confocal unit (CSU-X1, Yokogawa Electric, Tokyo, Japan), a cooled CCD camera (iXon DV897, Andor Technology, Belfast, U.K.), an upright microscope with a water-immersion objective (16×, Nikon), and image acquisition software (SOLIS, Andor Technology, Belfast, U.K.). Fluorophores were excited at 488 nm with an argon-krypton laser (1.5—1.8 mW) and visualized through a 507 nm long-pass emission filter. Spike-triggered calcium signals were semiautomatically detected with custom-written software in Visual Basic Version 6.0 (Microsoft, Seattle, WA, U.S.A.) and inspected by eye. The fluorescence change was measured as $(F_t - F_0)/F_0$, where F_t is the fluorescence intensity at a given

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time point and F_0 is the average baseline across 10 s before and after time t.

Asynchrony Index To assess the synchrony of the neuronal activity, we applied normalized Shannon index (SI) as an asynchrony index. SI is a measure of diversity given by $SI=-\sum_i (k_i/K)\log_2(k_i/K)$, where K is the total number of observations (here, calcium events detected by fMCI), and k_i is the number of observations in time window i (bin: 1 frame=100 ms). When all the observations occurred in a single time window (*i.e.*, complete synchronization), SI is zero. SI was normalized to the highest (SI_{max}) and the lowest (SI_{min}) value, which were obtained by rearranging all events in the dataset.[12] We then defined the "asynchrony index" as $(SI-SI_{min})/(SI_{max}-SI_{min})$. This normalization is required to compare data across specimens with varying numbers of neurons. This was detailed in the ref. 12. We reported the averaged data as the means±standard deviation.

Synchronous Spike Pairs Synchronous spike pairs (SSPs), *i.e.*, pairwise corrections, were used to further confirm the change of synchrony in the network.[13] In our datasets, the numbers of spontaneous spikes were often insufficient to precisely calculate the correlation coefficient. In point-process datasets like ours, in general, SSPs provides a better estimation of the pairwise similarity. SSPs, defined here as any pairs of spikes that concurred in two neurons, were detected with a time window of 100, 200, 300, or 400 ms time window (jitter) in the raw and surrogate data. Surrogate data were obtained by randomly shuffling the timings of calcium events within a cell. The process was repeated 20 times and was averaged to obtain the 'chance' numbers of SSPs in the surrogate data. Ratio of SSPs in the original data to their surrogate data (termed "Saliency Index") was calculated for control and OGD time window and compared. We reported the averaged data as the means±standard deviation.

RESULTS

Asynchronous Neuronal Activity Observed under OGD We simulated ischemia by perfusing organotypic hippocampal slice cultures with aCSF bubbled with 95% N_2–5% CO_2 without glucose, a commonly used oxygen-glucose deprivation (OGD) protocol. CA1 neuronal activity was captured as a somatic Ca^{2+} transient using fMCI. The timings of the calcium events or spike-like changes of Ca^{2+} fluorescence intensity, which reflect neuron firing, were determined for each cell. Because OGD application may change the intracellular ion balance, we first assured that calcium events detected by fMCI truly represent neuron firing even under OGD conditions. We carried out cell-attached recordings simultaneously with fMCI (Figs. 1A, B) and confirmed that calcium events detected by fMCI were consistent with firing spikes detected by cell-attached recording under OGD conditions.

In Fig. 1C, we visualized the neuronal network activity under OGD in the form of raster plot and histogram. Each dot in the raster plot represents the calcium event in individual cells, which is organized in the histogram according to the time course. When OGD was applied, the total number of calcium events per minute gradually increased to reach a peak and eventually ceased after a few minutes. The average activity peak latency after the OGD onset was 9.1±6.7 min,

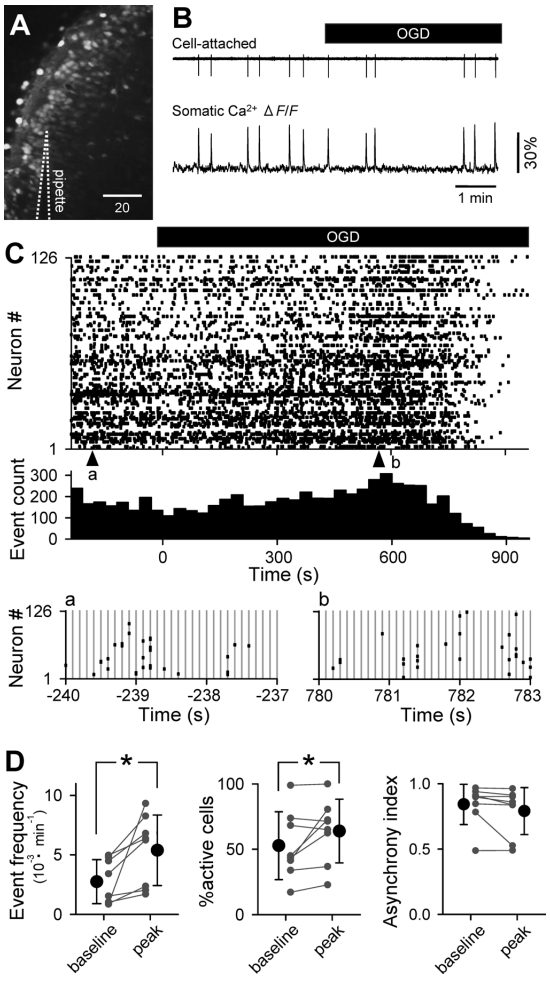

Fig. 1. CA1 Neuronal Activity Remains Asynchronous under OGD

(A) Representative image of OGB-1-loaded CA1 neurons. Dot indicates the shadow of a patch-clamp pipette used for cell-attached recording. (B) Cell-attached recordings (top) reveal that fMCI-detected somatic calcium events (bottom) worked as a faithful reporter of neuronal firing even under OGD conditions (periods indicated by the closed bar). (C) Top: representative raster plot, in which each dot indicates the time of a single calcium transient elicited by action potential of the corresponding neuron (numbered in the ordinate). Extracellular solution was switched from normal to OGD-aCSF at 0 min (closed bar). Middle: histogram of the level of network activity, each 30-s window of which the raster plot was vertically collapsed for activity summation. The entire neuronal activity peaked at 9 min. Bottom: the enlarged raster plots are the representative 3 s from the 'baseline' and 'peak' periods (indicated by arrowheads). The grids show the bin of 100 ms used in the calculation of asynchrony index in Fig. 1D. (D) Parameters show that under OGD, neuronal network activity increased but remained asynchronous (paired t-test, $p<0.05$, $n=8$ slices). Asynchrony index refers to normalized Shannon index, which expresses the degree of asynchrony at one being the maximal asynchronicity.

whereas the time from the peak to the decline of the enhanced activity (defined as the time giving the minimal event count after the peak) was 7.3±2.1 min ($n=8$ slices).

The characteristic of this enhanced activity was assessed using three parameters, *i.e.*, event frequency, %active cells, and asynchrony index (Fig. 1D). The 3-min period around the time that gave the maximal event count was defined as the 'peak' period and was compared with the control period of 3 min before the OGD onset, referred to as the 'baseline' period. The mean number of events per minute per cell (event frequency) and the percentage of cells that showed calcium events against the total recorded neuron population (%active cells) both showed a significant increase when OGD was applied (paired t-test, $p<0.05$, $n=8$ slices). This change implicates the increase in the overall excitability of

the recorded network, contributed both by the increase of active cells and the excitability of each cell. In contrast to this evident change in the network, the OGD application induced no change in the asynchrony index given by normalized Shannon index along the time course, 1 expressing maximum asynchrony (see Materials and Methods). This indicates that the degree of asynchrony maintained against the enhancement of the overall network activity. As shown in individual data in Fig. 1D, the asynchronous index varied from slice to slice, and some data exhibited a decrease in asynchronicity after OGD. This may be due to spontaneous fluctuation and occasional synchrony in baseline activity observed in organotypic slice cultures,[13,14] which could not be eliminated because we defined the measure period uniformly.

Confirmation of the Asynchrony under OGD The asynchrony index in the above analysis has been utilized in past reports as a sensitive and reliable parameter that reflects the degree of synchronicity of neuron networks.[12,15] But for this particular state we focused on, there is a possibility that we may have overlooked a slight difference in synchrony that this parameter was not sensitive enough to capture.

In order to examine this possibility, we compared SSPs, which is a more conventional and direct way to analyze synchrony.[13] The number of synchronized spike pairs was counted for the control and OGD period as defined above. To consider the increase in the event frequency and active cells, we shuffled the event timings in each period and counted the SSPs. The ratio of the SSPs in the original data and surrogate data were termed saliency index as shown in Fig. 2A. Saliency index shows how much the network is synchronized compared to expectation.

Consistent with the asynchrony index, the saliency index did not show significant difference between control and OGD period (Fig. 2B). To eliminate the possibility of fluctuation in the detection of spike events, the saliency index within the time window of 100, 200, 300, and 400 ms were compared, yet no significant differences were found. Thus we conclude that the sustainment of the asynchrony found by the comparison of asynchrony index is a definitive phenomenon also supported by the comparison of SSPs.

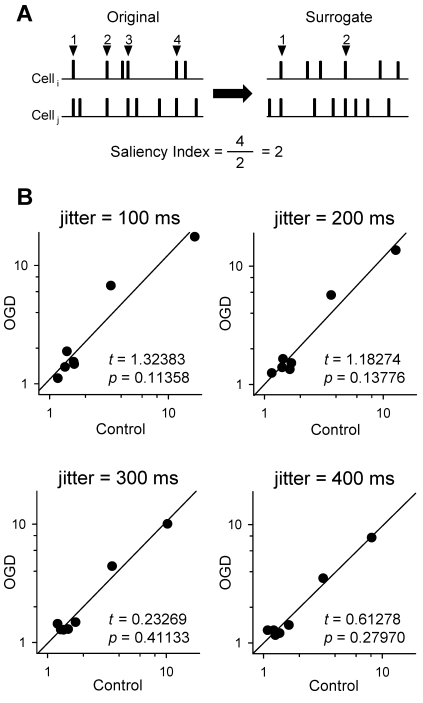

Fig. 2. The Maintenance of Asynchrony Is Confirmed by Comparison of SSPs

(A) A schematic drawing of the calculation of saliency index. The ratio of SSPs counted in original and surrogate data was compared in control and OGD period as saliency index. (B) Saliency index calculated allowing various time window of synchrony (jitter) were compared. In any case, no significant difference was found (paired t-test, $p<0.05$, $n=8$ slices), indicating that the network remained asynchronous under application of OGD.

DISCUSSION

By applying OGD to organotypic hippocampal slice cultures, we visualized how the neuronal network activity changes after energy deprivation. We found that the network showed a transient enhanced activity, distinct in a way that it remains asynchronous. We also confirmed this by the comparison of SSPs between control and OGD period. Hyperexcitation has been observed in other neuropathological state, and is also an expected feature of energy deprivation, as stated in many single-cell level molecular and pathological reports. But the response of the network as a whole was of surprise, as the network showed an apparent increase in excitability, but maintained an asynchronous state, in contrast to hyperthermia and epilepsy in which it shows highly synchronized activity. [15,17]

What makes it possible for the network to maintain the asynchronously enhanced activity? Studies on neural network simulation have shown that an asynchronous state with a high firing rate is achieved by strong inhibition.[16] Homogeneously increased excitability of nearly all excitatory and inhibitory neurons, as seen in high temperature conditions, induces synchrony,[15] indicating that stronger inhibition is needed to maintain the asynchronous state. During cortical UP-state where active but asynchronous state is achieved, the balance and timing of excitatory and inhibitory conductances is kept constant against a large change in the total membrane conductance.[18,19] Thus, for the hyperexcitation and asynchrony to co-exist within the network, the balance between excitation and inhibition must be maintained firmly with strong enough inhibition to restrain the excitation from running out of control.

Is it possible for the network to maintain its balance under energy deprivation? In simulated ischemic conditions, depolarization of presynaptic neurons and the change in intracellular ionic components lead to enhanced release and reduced uptake of glutamate,[1,3,4] which both enhance the excitation within the network. But at the same time, the decreases in intracellular ATP and pH enhance the synthesis and suppress the degradation of γ-aminobutyric acid (GABA), increasing the extracellular GABA concentration.[2] Furthermore, adenosine release is also known to be enhanced during *in vitro* ischemia, inhibiting the glutamatergic transmission.[5] Our observations, combined with these studies, suggest the possibility that at least for our observation period of tens of minutes, the inhibition is maintained (or even further enhanced) in balance with excitation, preventing it from inducing synchrony. This idea is also consistent with the observation of neuronal hyperpolarization under energy deprivation,[20] which is assumed to have a neuroprotective effect.

Which of the above reported responses contribute to maintaining asynchrony and whether the asynchrony we observed represents neuroprotective effect needs further investigation, but it should be noted that maintenance of asynchrony under pathologic states is quite characteristic, representing the singularity of neuronal network operation. The importance of observing the effect of disorders at the level of network should be emphasized; energy deprivation as in cerebral ischemia is commonly thought to bring about hyperexcitability, but its characteristic activity state could not be predicted from single-cell level studies. This network-based approach allows us to integrate the individual results obtained from cell molecular studies, giving an insight into "what is actually going on as a whole", which is undoubtedly an important and useful material in therapeutic decision-making and pharmaceutical approach.

Acknowledgement This work was supported in part by Grant-in-Aids for Science Research (No. 22650080 and No. 22680025) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by the Mochida Memorial Foundation for Medical and Pharmaceutical Research.

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