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Detection of synchronized burst firing in cultured human induced pluripotent stem cell-derived neurons using a 4-step method

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ABSTRACT

Human induced pluripotent stem cell-derived neurons are promising for use in toxicity evaluations in nonclinical studies. The multi-electrode array (MEA) assay is used in such evaluation systems because it can measure the electrophysiological function of a neural network noninvasively and with high throughput. Synchronized burst firing (SBF) is the main analytic parameter of pharmacological effects in MEA data, but an accurate method for detecting SBFs has not been established. In this study, we present a 4-step method that accurately detects a target SBF confirmed by the researcher's interpretation of a raster plot. This method calculates one set parameter per step, in the following order: the inter-spike interval (ISI), the number of spikes in an SBF, the inter-SBF interval, and the number of spikes in an SBF again. We found that the 4-step method is advantageous over the conventional method because it determines the preferable duration of an SBF, accurately distinguishes continuous SBFs, detects weak SBFs, and avoids false detection of SBFs. We found also that pharmacological evaluations involving SBF analysis may differ depending on whether the 4-step or conventional threshold method is used. This 4-step method may contribute to improving the accuracy of drug toxicity and efficacy evaluations using human induced pluripotent stem cell-derived neurons.

1. Introduction

Human induced pluripotent stem cell (iPSC)-derived neurons are promising for evaluating the toxicity of pharmacological agents in nonclinical studies [1,2]. One of the major adverse events affecting the central nervous system observed during clinical trials is convulsions [3]. The evaluation of drugs for the potential to cause convulsions requires an assay system that can measure the electrophysiological functions in neuronal networks. Multi-electrode array (MEA) systems have recently attracted attention for use in evaluating the convulsion potential of a drug because they noninvasively measure the electrophysiological activity of neural networks at multiple sites in a high-throughput manner. We previously reported the effectiveness of MEA measurements for evaluating responses to pharmacological agents, including convulsants, in human iPSC-derived neurons [4–6]. MEAs have been used successfully also to evaluate drug responses in human iPSC-derived cardiomyocytes for the screening of compounds with QT prolongation and proarrhythmic potential [7,8].

To evaluate neurotoxicity using MEA data, appropriate evaluation parameters must be derived. Synchronized burst firings (SBFs) are an important index of epileptiform activity and pharmacological effects in cultured neuronal networks. The SBF analysis parameters include the number of SBFs, the SBF duration, and the number of spikes in an SBF [4,9–12]. While accurate detection of SBFs is essential to this procedure, an accurate SBF detection method has not been established. In many analyses, the threshold is set using the histogram of the spike time stamp obtained with

Abbreviations: hiPSC, human induced pluripotent stem cell; MEA, multi-electrode array; SBF, synchronized burst firing; DIV, days *in vitro*; WIV, weeks *in vitro*; TIT, tohoku institute of technology.

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multiple electrodes to detect SBFs [13–17]; however, this method results in inaccurate data. For example, even if the number of SBFs is assessed correctly, the accurate acquisition of the duration and number of spikes in an SBF at the same time is difficult.

Present SBF detection methods include those based on the inter-spike interval (ISI), the number of spikes in an SBF, and the number of active electrodes [18–28]. However, no method can determine the precise duration of an SBF, distinguish SBFs that occur continuously, or detect small-scale SBFs.

In this study, we develop a novel SBF detection method that can accurately detect SBFs. This method is carried out in 4 steps, allowing the sequential determination of 4 parameters.

2. Materials and methods

2.1. Culture of hiPSC-derived cortical neurons

Human induced PSC-derived cortical neurons (hiPSCs) (XCL-1, XCell Science Inc., Novato, CA, USA) were cultured at 3.0×10^5 cells/cm^2 on 64-channel MEA chips (MED-P515A; Alpha Med Scientific, Osaka, Japan) coated with polyethyleneimine (Sigma) and laminin-511 (Nippi). For the culture on MEAs, a φ3.4-mm glass ring was placed in the middle of the MEA probe at the location of the electrode array, and cell suspensions were seeded inside the ring. After 1 h, neural maturation basal medium (NM-001-BM100, XCell Science Inc.) supplemented with neuron maturation supplement A (NM-001-SA100, XCell Science Inc.) and 100 U/mL penicillin/streptomycin (168–23191, Wako, Richmond, VA, USA) was applied around the ring, and the ring was removed carefully. After 8 days of culture, the medium was replaced with BrainPhys neuronal medium with SM 1 neuronal supplement (STEMCELL Technologies, Vancouver, BC, CAN). Human iPSC-derived astrocytes (XCL-1, XCell Science) were seeded at 3.0×10^4 cells per well. Human iPSC-derived neurons (ax 0019, Axol Bioscience Inc., Cambridge, UK) were cultured at 5.0×10^8 cells/cm^2 on 16-channels per well across 24-well MEA plates (Alpha Med Scientific) coated with ready set (ax0052, Axol Bioscience) and sure bond (ax0041, Axol Bioscience). Human iPSC-derived astrocytes (ax0084, Axol Bioscience) were seeded at 3.0×10^4 cells per well. After 21 days of culture, the medium was replaced with BrainPhys neuronal medium with SM 1 neuronal supplement (STEMCELL Technologies). The cultures were grown at 37 °C in a 5% CO_2/95% air atmosphere. Half of the medium was exchanged every 4 days.

2.2. Extracellular recording

The extracellular signals of spontaneous firings were acquired at 37 °C under a 5% CO_2 atmosphere using a 24-well MEA system (Presto; Alpha Med Scientific) and a 64-channel MEA system (MED64-Basic; Alpha Med Scientific) at a sampling rate of 20 kHz/channel. Signals were low-cut filtered at 100 Hz and stored on a personal computer. Spontaneous firing was recorded for 10 min in a medium and an artificial cerebrospinal fluid (ACSF). The chemical composition of the ACSF (in mM) is as follows: NaCl, 124.0 mM; KCl, 3.0 mM; $CaCl_2$, 2.0 mM; $MgCl_2$, 0 mM, NaH_2PO_4, 1.25 mM; $NaHCO_3$, 26.0 mM; and glucose, 10.0 mM.

2.3. Pharmacological test

To determine whether the pharmacological results differ between the conventional and the 4-step SBF methods, we administered the convulsant drug 4-aminophyrisine (4-AP), a K^+ channel blocker (0, 0.3, 1, 3, 10, and 30 μM) (275875, Sigma–Aldrich). For the conventional method, the 2 threshold values were set to above and below, respectively, 120 and 50 Hz (vehicle, 0.1, 1, and 3 μM 4-AP), 60 and 20 Hz (10 μM 4-AP), and 35 and 10 Hz (30 μM 4-AP). Spontaneous firing was recorded for 10 min at each concentration. The cultures were kept at 37 °C under a 5% CO_2 atmosphere between the recordings and drug administration.

2.4. Immunocytochemistry

The sample cultures were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) on ice (4 °C) for 10 min, followed by fixing in methanol on ice (−20 °C) for 10 min. The fixed cells were incubated with 0.2% Triton X-100 in PBS for 5 min, followed by preblock buffer (0.05% Triton-X and 5% goat serum in PBS) at 4 °C for 1 h, and finally with preblock buffer containing a specific primary antibody (rabbit anti-β -tubulin III, 1:1000) (T2200, Sigma–Aldrich) at 4 °C for 24 h. Immunolabeling was visualized by incubation in an appropriate secondary antibody anti-rabbit 488 Alexa Fluor (A21206, Thermo Fisher Scientific, 1:1000 in preblock buffer) for 1 h at room temperature. The cell nuclei were counterstained using 1 μg/mL Hoechst 33258 (H341, DOJINDO) for 1 h at room temperature. The stained cultures were washed twice in preblock buffer (5 min/wash), were rinsed twice using PBS, and were viewed using a confocal microscope (TCS SP8, Leica). The image intensity was adjusted using ImageJ software (NIH).

2.5. Data analyses

Electrophysiological activity was first detected using Presto and Mobius software (Alpha Med Scientific). A spike was counted if the recorded signal exceeded a threshold of ±5 σ, where σ is the standard deviation of the baseline noise during quiescent periods. The SBFs were detected using the 4-step method shown in Fig. 1. The duration and number of spikes in an SBF were calculated using MATLAB. The conventional two-threshold SBF detection method with a spike histogram was also used [13]. Spike histograms were generated using a bin size 50 ms and smoothing of 3 bin. The upper and lower thresholds to detect the start and finish of an SBF were 800 and 600 spikes/s, respectively. All the data are presented as the mean ± standard error. Statistically significant differences were determined using the two-tailed, paired Student's *t*-test.

3. Results

3.1. Spontaneous firings in cultured hiPSC-derived cortical neurons on MEA chips and SBF detection using conventional threshold method

To develop the synchronized burst firing detection method, we recorded the spontaneous firings in cultured hiPSC-derived cortical neurons on MEA chips. The hiPSC-derived cortical neurons were grown on MEA chips as shown in Fig. 1A and they survived in good health for over 200 days. Spontaneous firings were observed over 2 or 3 weeks at several electrodes, and SBFs were observed over 5 weeks. Fig. 1A–b shows the waveform of typical spontaneous firings at 115 days *in vitro* containing weak and strong SBFs, as shown in red squares. Fig. 1B shows SBF detection using the conventional two-threshold method, in which the upper and lower thresholds determine the start and finish, respectively, of an SBF. The intersection between the smoothing line and the threshold value was identified and was used to determine the start and end of an SBF (Fig. 1B–b). The upper and lower thresholds were set to 800 and 550 spikes/sec, respectively, to allow SBF detection with the duration indicated by the orange arrow in Fig. 1B–a. An attempt to detect an SBF of the preferred duration, indicated by the orange arrow, will erroneously detect the element indicated by the black arrow (Fig. 1B–a). Detection errors occurred, including the

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