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Long-term electrophysiological activity and pharmacological response of a human induced pluripotent stem cell-derived neuron and astrocyte co-culture

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ABSTRACT

Human induced pluripotent stem cell (hiPSC)-derived neurons may be effectively used for drug discovery and cell-based therapy. However, the immaturity of cultured human iPSC-derived neurons and the lack of established functional evaluation methods are problematic. We here used a multi-electrode array (MEA) system to investigate the effects of the co-culture of rat astrocytes with hiPSC-derived neurons on the long-term culture, spontaneous firing activity, and drug responsiveness effects. The co-culture facilitated the long-term culture of hiPSC-derived neurons for >3 months and long-term spontaneous firing activity was also observed. After >3 months of culture, we observed synchronous burst firing activity due to synapse transmission within neuronal networks. Compared with rat neurons, hiPSC-derived neurons required longer time to mature functionally. Furthermore, addition of the synapse antagonists bicuculline and 6-cyano-7-nitroquinoxaline-2,3-dione induced significant changes in the firing rate. In conclusion, we used a MEA system to demonstrate that the co-culture of hiPSC-derived neurons with rat astrocytes is an effective method for studying the function of human neuronal cells, which could be used for drug screening.

1. Introduction

Human induced pluripotent stem cells (hiPSCs) differentiation techniques and culture methods are important evaluation models and an alternative to animal testing for drug discovery screening, toxicity testing, and elucidating disease mechanisms [1–3]. Neuronal cells can be generated from hiPSCs, providing a very important alternative to studies of humans and model organisms, to facilitate a better understanding of the mechanisms of neurological diseases and identifying novel therapeutic avenues [4,5]. However, hiPSC-derived neurons cultured using traditional two-dimensional culture methods lack adequate maturation, and evaluation methods focusing on the function of cultured hiPSC-derived neurons have not been established. Additionally, using glial cell humoral factor in cultured neuronal cells increases the number of living cells [6,7]. We previously demonstrated that achieving long-term single cell culture is possible by adding glial cell humoral factor to rat hippocampal neurons [8]. Additionally, synapse maturation was detected in the presence of physical contact with astrocytes [9]. Furthermore, results suggest that synaptic transmission between presynaptic, postsynaptic, and glial cells was enhanced by astrocytes in neuronal networks [10–12], which could be a very important step in mature cultured hiPSC-derived neuron production. A well-known traditional method for measuring the function of cultured neurons is the patch clamp method using a glass micropipette, used to analyze the function of hiPSC-derived neurons [13]. However, this method is invasive and not suitable for long-term measurement. In contrast, using a multi-electrode array (MEA) system facilitates non-invasive, real-time, multi-point measurement of the activity of cultured neurons [6,14–16]. Therefore, this may be a suitable method for studying the function of hiPSC-derived neurons. The MEA system has been used to evaluate the plasticity of rat and mouse neuronal networks [15], neuronal diseases [17], and drug responsiveness. A study showed that the MEA system is useful for toxicity evaluation and other analytical methods with hiPSC-derived cardiomyocytes [18–20]. Therefore, the MEA system could be useful for studying hiPSC-derived neurons.

Abbreviations: CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DIV, days in vitro; hiPSC, human induced pluripotent stem cell; MEA, multi-electrode array; PBS, phosphate-buffered saline.

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Here, we used the MEA system to investigate the functional effects of co-culturing rat astrocytes with hiPSC-derived neurons on their long-term spontaneous activity and drug responsiveness.

2. Materials and methods

2.1. Planar MEA chip

To evaluate the long-term culture of hiPSC-derived neurons, we used a planar MEA measurement system (Alpha Med Scientific, Japan). The MEA chips were produced on a glass slide, comprising 64 electrodes (50 × 50 μm) with 150-μm spacing in an 8 × 8 grid arrangement (MED-P515A; Alpha Med Science). Each recording terminal surface was coated with Pt/Pt-black to reduce impedance.

2.2. hiSPC-derived neurons

hiPSC-derived neurons [iCell neurons; Cellular Dynamics International (CDI) Inc., USA] were cultured on MEA chips. The MEA chips were coated with 0.05% polyethylenimine solution (Sigma–Aldrich) for 1 h at room temperature, washed four times with sterilized water, and left to dry overnight. Next, 5 μg/ml of laminin solution was added, and the chips were incubated at 37 °C in a 5% CO_2/95% air atmosphere. On the day that culture commenced, the laminin solution was removed and a φ3.4-mm glass ring was placed in the middle of the probes where the electrodes were located. The cells (90 μl) were seeded inside the rings (density, 1.3×10^6 cells/ml) and incubated for 1 h. Culture medium was applied around the rings and were removed carefully. The cultures were grown at 37 °C in a 5% CO_2/95% air atmosphere.

2.3. Humoral factor derived from rat astrocytes and rat astrocyte co-culture

iCell neuron maintenance medium (NRM-100-121-001, CDI, Inc., USA) was supplemented with iCell neuron medium supplement (NRM-100-031-001, CDI, Inc., USA) and 100 U/ml penicillin/streptomycin (Invitrogen). To evaluate the pharmacological response and long-term spontaneous activity characteristics, we tested iCell neurons in three different culture conditions: (i) conventional culture conditions using samples with maintenance medium culture; (ii) conventional culture conditions with the addition of rat astrocyte-derived humoral factor; and (iii) with the addition of rat astrocyte-derived humoral factor and rat astrocytes.

To obtain the rat astrocytes, we cultured rat primary hippocampal neurons for one month in culture medium (Neurobasal medium) supplemented with 2% v/v B27 supplement, 10% v/v fetal bovine serum, 100 U/ml penicillin/streptomycin (all from Invitrogen), and 0.074 mg/ml L-glutamine (Sigma). The rat astrocytes were seeded (density, 1×10^3 cells/MEA chip) and co-cultured with hiPSC-derived neurons. The humoral factor was obtained from the same culture medium used to produce the astrocytes.

2.4. Immunocytochemistry

The samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) on ice (4 °C) for 10 min, followed by 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 in preblock buffer containing a specific primary antibody at 4 °C for 24 h. The primary antibodies used were rabbit anti-GFAP (Millipore) for the specific labeling of astrocytes, mouse anti-β-tubulin III (Sigma–Aldrich) for the specific labeling of neurons, and rabbit anti-synaptophysine for the specific labeling of presynapses. All antibodies were dissolved at 1:1000 in preblock buffer. Secondary antibodies (anti-mouse 488 Alexa Fluor and anti-rabbit 546 Alexa Fluor, Molecular Probes; 1:1000 in preblock buffer) and 1 μg/ml Hoechst 33258 were used for nuclear labeling, which were applied for 1 h at room temperature. The stained cultures were washed twice using preblock buffer (5 min each), rinsed twice with PBS, and viewed using an electron multiplying CCD camera (iXon Ultra 897, Andor). The image intensity was adjusted using ImageJ software.

2.5. Extracellular recording

Spontaneous recordings were obtained for 10 min each week to determine whether the long-term spontaneous firing depended on the culture conditions. The extracellular signals detected by the MEA system were amplified using a 64-channel amplifier (SU-MED64; Alpha MED Science) and stored on a personal computer. A sampling rate of 20 kHz/channel was used. The cultures were kept in an incubator during the recordings. Firing analyses were performed using Mobius software (Alpha Med Scientific Inc.).

2.6. Pharmacological tests

To investigate pharmacological effects, we administered synaptic antagonists to hiPSC-derived neuronal networks on the MEA chips. The $GABA_A$ receptor antagonist bicuculline (Sigma–Aldrich) and the AMPA/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Sigma–Aldrich) were used at final concentrations of 10 and 30 μM in the medium, respectively. Spontaneous recordings were obtained for 60 min before treatment with bicuculline or CNQX and again after administration. A 10-min washout was performed in the medium prior to recordings. The cultures were kept in a CO_2 incubator between the recordings and washouts. To avoid medium changes and waiting for the effects of drugs, firing analyses were performed during the last 10 min of the 60-min period after drug administration.

2.7. Statistical analysis

All data were expressed as mean ± standard error, and the statistically significant differences were calculated using a two-tailed paired Student's *t*-test.

3. Results

3.1. Morphological effects on hiPSC-derived neurons and rat astrocyte co-culture

To investigate the electrophysiological activity of hiPSC-derived neurons, we tested three different culture conditions: (i) hiPSC-derived neurons, (ii) co-culture of hiPSC-derived neurons and rat astrocyte-derived humoral factor, and (iii) co-culture of hiPSC-derived neurons, rat astrocyte-derived humoral factor, and rat astrocytes (Fig. 1A). Fig. 1B shows hiPSC-derived neurons cultured on top of a MEA chip (density, 1.3×10^6 cells/cm^2). To investigate hiPSC-derived neuron morphology when co-cultured with rat astrocytes and humoral factor, we immunostained the samples at 42 culture days in vitro (DIV). The astrocyte marker GFAP was negative in the first and second culture conditions (Fig. 1C-a), whereas it was positive when hiPSC-derived neurons were co-cultured with rat astrocytes, suggesting that culturing rat astrocytes using the MEA probe is possible (Fig. 1C-a). Furthermore, the rat astrocytes and hiPSC-derived neurons were in physical contact, which was demonstrated by the pseudopodial processes (Fig. S1).