Journal of Neuroscience Methods 293 (2018) 53–58

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

Short communication

Method of derivation and differentiation of mouse embryonic stem cells generating synchronous neuronal networks

Elena V. Gazina[a], Emma Morrisroe[a], Gunarathna D.C. Mendis[b], Anna E. Michalska[c], Joseph Chen[d], Christian M. Nefzger[d], Benjamin N. Rollo[a], Christopher A. Reid[a], Martin F. Pera[a,c], Steven Petrou[a,e,*]

[a] The Florey Institute of Neuroscience and Mental Health, Parkville, VIC 3052, Australia
[b] Department of Mechanical Engineering, The University of Melbourne, Parkville, VIC 3052, Australia
[c] Stem Cells Australia, Parkville, VIC 3052, Australia
[d] Department of Anatomy and Developmental Biology, Australian Regenerative Medicine Institute, Monash University, Clayton, VIC 3800, Australia
[e] Centre for Neural Engineering, The University of Melbourne, Parkville, VIC 3052, Australia

HIGHLIGHTS

- A refinement of a protocol for mouse ES cell differentiation is described.
- ES cell derived and primary neuron cultures were grown and functionally analysed using microelectrode arrays.
- At 35 days in culture ES cell differentiated neuronal networks recapitulated functional properties of primary cortical neuronal networks.
- ES cell neurons may serve as a viable alternative to primary neurons with implications for modelling of human disease.

ARTICLE INFO

Article history:
Received 2 May 2017
Received in revised form 11 August 2017
Accepted 14 August 2017
Available online 18 August 2017

Keywords:
Embryonic stem cells
Neuronal differentiation
Neuronal networks
Cortical neurons

ABSTRACT

Background: Stem cells-derived neuronal cultures hold great promise for *in vitro* disease modelling and drug screening. However, currently stem cells-derived neuronal cultures do not recapitulate the functional properties of primary neurons, such as network properties. Cultured primary murine neurons develop networks which are synchronised over large fractions of the culture, whereas neurons derived from mouse embryonic stem cells (ESCs) display only partly synchronised network activity and human pluripotent stem cells-derived neurons have mostly asynchronous network properties. Therefore, strategies to improve correspondence of derived neuronal cultures with primary neurons need to be developed to validate the use of stem cell-derived neuronal cultures as *in vitro* models.
New method: By combining serum-free derivation of ESCs from mouse blastocysts with neuronal differentiation of ESCs in morphogen-free adherent culture we generated neuronal networks with properties recapitulating those of mature primary cortical cultures.
Results: After 35 days of differentiation ESC-derived neurons developed network activity very similar to that of mature primary cortical neurons. Importantly, ESC plating density was critical for network development.
Comparison with existing method(s): Compared to the previously published methods this protocol generated more synchronous neuronal networks, with high similarity to the networks formed in mature primary cortical culture.
Conclusion: We have demonstrated that ESC-derived neuronal networks recapitulating key properties of mature primary cortical networks can be generated by optimising both stem cell derivation and differentiation. This validates the approach of using ESC-derived neuronal cultures for disease modelling and *in vitro* drug screening.

Abbreviations: ESC, embryonic stem cell; MEA, microelectrode array; LIF, leukemia inhibitory factor; bFGF, basic fibroblast growth factor; DiffD, differentiation day; DM, differentiation medium; DiV, days *in vitro*.

* Corresponding author at: The Florey Institute of Neuroscience and Mental Health, 30 Royal Parade, Parkville, VIC, 3052, Australia.
E-mail address: spetrou@unimelb.edu.au (S. Petrou).

http://dx.doi.org/10.1016/j.jneumeth.2017.08.018
0165-0270/

1. Introduction

Neuronal cultures derived from induced human pluripotent stem cells have great potential to model neurological disorders and to be used for drug screening and cell transplantation-based therapy. However, the current derivation protocols produce human neurons with immature functional properties even after extended time in culture, which impedes their use requiring significant improvement of differentiation methods [e.g. (Amin et al., 2016; Odawara et al., 2016)]. Neuronal differentiation of mouse embryonic stem cells (ESCs) produces more mature neurons compared to the human culture. In addition, the availability of murine brain tissue allows direct comparison between primary and derived neurons, which cannot be easily made for the human cells. This provides an opportunity to select differentiation conditions that generate ESC-derived neurons with the properties matching those of primary neurons. Such ESC-derived neuronal cultures could be used to model neurological disorders and for *in vitro* drug screening, replacing currently used primary neuronal cultures and thus reducing the use of animals and experimental costs. In addition, the acquired knowledge may be useful for improving the differentiation protocols for human cultures.

Neurons have a fundamental ability to form networks generating spontaneous synchronised electrical activity *in vivo* and *in vitro*. The establishment of networks in *ex vivo* neuronal cultures can be monitored using microelectrode arrays (MEAs), a non-invasive method of measuring neuronal activity and synaptic signalling using electrodes embedded in a culture dish. Dissociated cortical neurons of embryonic or neonatal mice generate spontaneous spiking activity on MEAs after a few days *in vitro*. Over time the spikes convert into repetitive burst discharges, which are then synchronised over large fractions of the culture [e.g. (Lee et al., 2014; Sun et al., 2010)].

Neuronal cultures have been generated from mouse ESCs using a variety of methods. So far two differentiation strategies have been shown to generate functional neuronal networks: suspension culture of embryoid body-like aggregates, and co-culture of ESCs with bone marrow-derived stromal cells (Ban et al., 2007; Illes et al., 2014, 2009). The level of synchrony in the derived neuronal cultures was, however, significantly lower compared to the primary cortical and hippocampal cultures (Ban et al., 2007; Illes et al., 2014). Gaspard et al. developed adherent ESC culture conditions in morphogen-free medium that generate cortical neurons (Gaspard et al., 2009, 2008). This differentiation strategy reduces the complexity of neuronal induction compared to neurogenesis via embryoid bodies or co-culture with stromal cells; however, network properties of neurons derived using this method have not been examined.

In this study we derived ESCs from mouse embryos in serum-free medium and without feeder cells, and differentiated them using a modification of the method of Gaspard et al. (2009, 2008). We showed that network properties of the resulting neurons were highly similar to those of *ex vivo* cortical neurons from the same mouse line.

2. Materials and methods

All animal experiments were approved by the Florey Institute Animal Ethics Committee, and performed in accordance with the Prevention of Cruelty to Animals Act and the NHMRC Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.1. Derivation of ESCs

ESCs were derived from F1 hybrid C57BL/6J:129/SvImJ blastocysts using a previously described protocol (Czechanski et al., 2014) with the following modifications. The ESCs were derived and propagated without using feeder cells, on gelatin-coated culture plates, in ESC medium containing KnockOut DMEM supplemented with 2 mM L-glutamine, 100 μM non-essential amino acid solution, 100 μM 2-mercaptoethanol, 1× penicillin/streptomycin, 1× N2 supplement, 1× B27 supplement, 1% KnockOut serum replacement, 1 μM PD0325901, 3 μM CHIR99021 and 10^3 U/ml murine leukemia inhibitory factor (LIF). PD0325901, CHIR99021 and LIF were purchased from Merck KGaA, all other reagents were from Thermo Fisher Scientific. The cells underwent 5 passages prior to differentiation.

2.2. Neuronal differentiation of ESCs

The differentiation protocol was adapted from the protocol of Gaspard et al. (2009) as follows. ESCs derived from a single embryo were plated into gelatin-coated 25 cm^2 flasks in the ESC medium at three densities: 2500, 5000 and 10,000 cells/cm^2. After 24 h the medium was changed to differentiation medium (DM) containing DMEM/F12 with GlutaMAX (Thermo Fisher Scientific) supplemented with 1× N2 supplement, 1× B27 supplement without vitamin A (Thermo Fisher Scientific), and 20 μg/ml human insulin (Merck KGaA). This was considered differentiation day (DiffD) 0. The cells were cultured for 11 days with daily medium changes, after which they were dissociated using 0.05% trypsin/0.5 mM EDTA and plated onto 24-well MEA plates (12 electrodes/well, Multichannel Systems), coated with polyethyleneimine/laminin (310/0.31 μg/cm^2), in DM supplemented with 20 ng/ml of basic fibroblast growth factor (bFGF, R&D Systems). Four plating densities were tested: 1.25×10^5, 2.5×10^5, 5×10^5 and 10^6 cells/cm^2. 48 h later bFGF was removed and the cells were cultured in DM until DiffD 44. Approximately 90% of spent medium was replaced every 48 h, without exposing cells to the air. MEA recordings were conducted every 2–3 days starting from DiffD 18, from 6 wells for each combination of plating densities.

2.3. Primary cortical culture

Cells were dissociated from the cortices of sixteen F1 hybrid C57BL/6J:129/SvImJ mice at postnatal days 0–2, and cultured on polyethyleneimine/laminin-coated 24-well MEA plates (132 wells total) as described previously (McSweeney et al., 2016) with the following modifications. The cells were plated at the density of 1.17×10^6 cells/cm^2 in minimal essential medium (Merk KGaA) supplemented with 6 g/l D-glucose (Merk KGaA), 1× penicillin/streptomycin, 9 mM HEPES-NaOH, pH 7.3 and 10% fetal bovine serum (Thermo Fisher Scientific). Three hours after plating, the medium was replaced with Neurobasal-A medium supplemented with 2 mM GlutaMAX, 1× B27 supplement, 1× penicillin/streptomycin and 9 mM HEPES-NaOH, pH 7.3. Approximately 90% of spent medium was replaced every 2–3 days. Cytosine arabinoside (5 μM, Merk KGaA) was added to the medium at 3 days *in vitro* (DiV) for 48 h to stop glial proliferation. MEA recordings were conducted at 14, 21 and 28 DiV.

2.4. MEA recordings and data analysis

MEA plates were placed on a Multiwell-MEA headstage (Multichannel Systems), into an enclosed recording chamber kept at 37 °C. The recordings were performed for 11 min at 20 kHz. Data acquisition was carried out using Multiwell-Screen software (Multichannel Systems). The signals were filtered (high-pass 1 Hz, low-pass 3.5 kHz) using inbuilt hardware filters. Voltage signals of each electrode were further high-pass filtered at 300 Hz, and 26 network characteristics were extracted in terms of firing and bursting parameters using custom MATLAB scripts. Spikes were detected as