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NSM-7669; No. of Pages 9

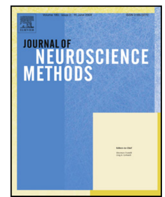
ARTICLE IN PRESS

Journal of Neuroscience Methods xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

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



Research paper

A three-dimensional neural spheroid model for capillary-like network formation

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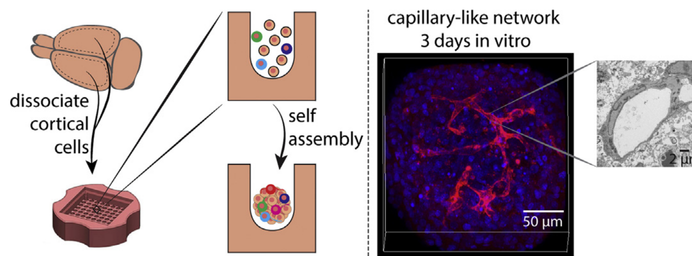
HIGHLIGHTS

- A three-dimensional neural spheroid model to study capillary-like structure formation is proposed.
- Endothelial cells within mixed cortical spheroids assembled into lumenized capillary-like structures.
- Capillary-like networks associated with relevant basement membrane proteins and neural cell types.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 29 April 2016
Received in revised form 19 January 2017
Accepted 24 January 2017
Available online xxx

Keywords:
Brain
Cortex
Vasculogenesis
in vitro
Spheroid
Neurovascular unit
Capillary-like network
Cellular self-assembly
Endothelial cells

ABSTRACT

Background: *In vitro* three-dimensional neural spheroid models have an *in vivo*-like cell density, and have the potential to reduce animal usage and increase experimental throughput. The aim of this study was to establish a spheroid model to study the formation of capillary-like networks in a three-dimensional environment that incorporates both neuronal and glial cell types, and does not require exogenous vasculogenic growth factors.
New method: We created self-assembled, scaffold-free cellular spheroids using primary-derived postnatal rodent cortex as a cell source. The interactions between relevant neural cell types, basement membrane proteins, and endothelial cells were characterized by immunohistochemistry. Transmission electron microscopy was used to determine if endothelial network structures had lumens.
Results: Endothelial cells within cortical spheroids assembled into capillary-like networks with lumens. Networks were surrounded by basement membrane proteins, including laminin, fibronectin and collagen IV, as well as key neurovascular cell types.
Comparison with existing method(s): Existing *in vitro* models of the cortical neurovascular environment study monolayers of endothelial cells, either on transwell inserts or coating cellular spheroids. These models are not well suited to study vasculogenesis, a process hallmarked by endothelial cell cord formation and subsequent lumenization.

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http://dx.doi.org/10.1016/j.jneumeth.2017.01.014
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Please cite this article in press as: Boutin, M.E., et al., A three-dimensional neural spheroid model for capillary-like network formation. J Neurosci Methods (2017), http://dx.doi.org/10.1016/j.jneumeth.2017.01.014

Conclusions: The neural spheroid is a new model to study the formation of endothelial cell capillary-like structures *in vitro* within a high cell density three-dimensional environment that contains both neuronal and glial populations. This model can be applied to investigate vascular assembly in healthy or disease states, such as stroke, traumatic brain injury, or neurodegenerative disorders.

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1. Introduction

Endothelial progenitor cells coordinate in the process of vasculogenesis to develop the embryonic vascular plexus. From this complex vascular network, endothelial cells (ECs) undergo angiogenesis and sprout into previously avascular tissues, including the neuroectoderm (Xu and Cleaver, 2011). Central nervous system vasculature matures into a specialized system, coordinating complex interactions between diverse cell types and scaffolding proteins to create the blood-brain barrier (BBB), which protects the sensitive neural environment. The timeline of rodent brain vascularization begins around embryonic day 11 (E11), a stable BBB is present at E15, and ECs continue to proliferate postnatally as the brain grows and develops (Robertson et al., 1985; Breier et al., 1992; Hagan and Ben-Zvi, 2015). Historically, the BBB is thought to be composed of three main cell types: (1) ECs, which utilize tight junction protein complexes to form a primary barrier and limit paracellular diffusion; (2) pericytes, which embed in the vascular basement membrane and assist in BBB function; and (3) astrocytes, which extend endfeet around vessels, deliver nutrients, and regulate tight junction expression. Basement membrane protein layers promote vessel stability and accumulate necessary growth factors (Obermeier et al., 2013). Neurons play a role in the vascular environment through an incompletely understood process called neurovascular coupling, in which neurons alter blood flow based on local metabolic requirements (Hamel, 2006; Moore and Cao, 2008; Lecrux and Hamel, 2011).

Neurovascular dysfunction is linked to many diseases, such as stroke, traumatic brain injury (TBI), and Alzheimer's Disease (AD). In these pathologies, vascular disruption results in a loss of brain tissue homeostasis and neurovascular coupling, leading to further tissue injury and disease progression (Girouard, 2006; Abbott et al., 2010; Shlosberg et al., 2010; Zhao et al., 2015). In the days following insults such as stroke or TBI, hypoxia and disruption of blood flow can stimulate the creation of new vasculature, termed neovascularization. The creation of new vasculature is associated with improved neurological recovery after brain injury (Xiong et al., 2010; Vallon et al., 2014; Prakash and Carmichael, 2015). In AD, vascular growth and remodeling are inhibited by the accumulation of amyloid β, which sequesters vascular endothelial growth factor (VEGF) and exerts anti-angiogenic activity (Vallon et al., 2014). Understanding neovascularization in these diseases is essential for the development of effective therapeutics to improve neurological outcome.

In vitro models of the specialized neurovascular environment are imperative for advancing understanding of healthy and pathological states, and for developing therapeutics. While many *in vitro* models exist to study insults to the established BBB, few are suitable for studying neurovascular capillary formation. The most common *in vitro* BBB model is the transwell assay, in which ECs are seeded on a transwell insert, and assessed as different cell types, toxins, and/or growth factors are placed on either side of the transwell membrane (Nakagawa et al., 2007; Osada et al., 2011; Xue et al., 2013). While the transwell model offers a practical format for studying *trans*-endothelial electrical resistance, ECs in two-dimensional (2D) culture often lose features necessary for BBB integrity (Calabria and Shusta, 2008; Urich et al., 2012). Additionally, few BBB models exist that incorporate the complex mixture of cell types found in the neurovascular unit.

Cells in three-dimensional (3D) culture establish cell–cell contacts in all directions and demonstrate more *in vivo*-like gene and protein expression, relative to 2D culture (Birgersdotter et al., 2005; Pampaloni et al., 2007; Page et al., 2013). A recent study showed that cells in a 3D BBB model expressed higher levels of cell adhesion molecules relative to transwell cultures, and exposed differences in receptor expression unobservable in the transwell model (Urich et al., 2013), demonstrating the importance of 3D platforms. We recently reported that 3D self-assembled spheroids composed of primary postnatal rat cortical cells have a brain-like tissue stiffness, mature neuronal electrophysiology, and markers of multiple neural cell types (Dingle et al., 2015). This model offers an *in vivo*-like cell density environment, without introducing artificial matrix materials.

In the present study our goal was to investigate whether this 3D neural model can be used to study the formation of capillary-like networks. We show that cortical ECs are capable of self-assembling into capillary-like networks within spheroids, without requiring exogenously introduced vasculogenic growth factors. Capillary-like networks are surrounded by basement membrane proteins, and interact with relevant neural cell types. Additionally, as a proof of concept experiment, we demonstrate that cortical spheroids created from mice with endogenously fluorescent ECs can be used to perform live-imaging of capillary-like networks. This model provides a 3D scaffold-free environment to study interactions between the complex cells of the neurovascular unit in an *in vitro* setting.

2. Materials and methods

2.1. Animal usage

All animal procedures were conducted in accordance with the guidelines established by the NIH and approved by Brown University's Institutional Animal Care and Use Committee. For studies using rats, CD rats (Charles River) were used.

For studies using endogenously fluorescent mice, cre expression in ECs was mediated via the mouse receptor tyrosine kinase (Tek, Tie2) promoter (Jax no. 004128) (Koni et al., 2001). These animals were crossed with Rosa-CAG-LSL-eNpHR3.0-eYFP-WPRE line 39 (Jax no. 014539) to enable expression of enhanced yellow fluorescent protein (eYFP) and halorhodopsin in Tie2-positive cells. While both eYFP and halorhodopsin were expressed in Tie2-positive cells, the goal of the current studies was to utilize eYFP for EC visualization.

2.2. Cell isolation and culture

Primary cortical tissue was isolated from postnatal day 1–3 rats or mice, and cells were isolated using a modified version of the protocol developed by BrainBits, as previously described (Dingle et al., 2015). Briefly, cortical dissection was performed in a buffer of Hibernate A (BrainBits) with 1X B27 supplement (Invitrogen), and 0.5 mM Gluta-Max (Invitrogen). Rat or mouse cortices were

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