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Neurovascular unit on a chip: the relevance and maturity as an advanced *in vitro* model

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The brain is a high-energy demanding organ, consuming around 20% of the metabolic energy generated. To fulfill this demand, cerebral blood flow (CBF) supplies oxygen and glucose continuously through the intricate network of cerebral blood vessels. Although for many years brain activity and blood flow were conceived as independent processes, MRI-based functional brain imaging demonstrated that there is a coupling between them, leading to the concept of the neurovascular unit (NVU) to reflect their interplay (Raichle and Mintun, 2006). The cerebrovascular system is far from being homogeneous throughout its structure; rather, each specific region of the brain presents multiple architectures. The NVU structure is divided into three main regions: cerebral arteries, including the middle cerebral artery. Middle cerebral artery gives rise to a second region, the pial arteries from the surface of the brain within the subarachnoid space. The endothelial cells (ECs) of pial arteries are covered by smooth muscle cells (SMCs) and are separated by a collagenous elastic lamina. From pial arteries, a subset of arterioles penetrates deep into the brain (penetrating arterioles) through the perivascular space, which limits glial cells. The perivascular space allocates several cell types; perivascular macrophages, mato, pial and mast cells. In this region, most external penetrating arterioles preserve a thin layer of SMCs and innervation. As arterioles get deeper into the brain, they become thinner, glial membrane and vascular basement membrane fuse occluding the perivascular space, innervation and SMCs disappear, which is ultimately substituted by pericytes in the microvascular capillaries, going to a less contractile vascularization. The collagen-rich matrix of pial arteries and brain capillaries are more abundant in proteoglycans and regulatory factors.

This structural diversity is important when we delineate the mechanism involved in CBF regulation through neurovascular coupling. Localized neuronal activity in a brain region triggers a vast vascular response known as hyperemia, which is balanced by feedback mechanisms according to the energy requirements of the tissue (Iadecola, 2017). For example, activation of glutamate receptors in post-synaptic neurons triggers an increment of intracellular Ca^{2+} that finally produces the release of vasodilators such as oxide nitric and prostanoids. Neurons can regulate the CBF but also astrocytes are able to trigger the same response (Iadecola, 2017). The interruption in the blood supply as in ischemic stroke or heart failure can lead to irreversible brain damage or even death. However, more subtle alterations of the CBF can lead to brain injury and cognitive impairment. Neurovascular uncoupling is linked to the onset and progression of several neurological disorders including neurodegenerative diseases (NDDs). In Alzheimer's disease, a reduction in CBF and attenuated hemodynamic responses to neural activation can already be detected in the pre-symptomatic phase of the disease. However, there is still a gap in understanding how these basic physiological findings translate to disease models and the identification of new relevant pharmacological targets.

Due to its role in regulating brain function and disease, the neurovasculature integration into more reliable *in vitro* models that adequately replicate brain anatomical features, cell-cell interactions, and blood flow, need to be developed. Organ-on-a-chip (OoC) is an ascending field providing versatile platforms to recreate physiological and pathological conditions in a spatiotemporal controlled approach. OoC offers an animal-free alternative to modulate different parameters such as configuration (sandwich, parallel, tubular, and vasculogenic), cell type, extracellular matrix, and shear stress, among others. In the last years, OoC has been used to reproduce the NVU (NVU-oC) for multiple applications from drug discovery to model NDDs (**Figure 1**). In models for drug testing and disease, cells of human origin are preferred. Primary cells retain more physiological information than cell lines. However, as embryonic stem cells and progenitor cells, they are subjected to ethical concerns and are difficult to isolate. Moreover, there are batch-to-batch differences, and they lose their original phenotype with passaging and culture. To overcome these limitations, human induced pluripotent stem cells (hiPSCs) capable of generating the multiple phenotypes of the NVU and, which can be disease-specific, have become a good alternative in NVU-oC models. Despite these advantages, hiPSCs differentiation is not straightforward and is subjected to variability. In 2015, Brown et al. developed one of the first NVU-oC consisting of a sandwich-like configuration, which included astrocytes, pericytes, and cortical neurons embedded in a 3D matrix of collagen type I in a brain compartment, while ECs were attached to the vascular space. Remarkably, they employed mostly human primary cells in addition to hiPSCs-derived cortical neurons. Moreover, they employed a system to monitor the transendothelial electrical resistance between ECs to evaluate the barrier permeability (Brown et al., 2015). Conversely, Koo et al. (2018) employed exclusively immortalized brain murine cells: ECs (bEnd.3), neuroblastoma (N2a), astrocytes (C8-D1A) and microglia (BV-2) also embedded into a collagen type I matrix. Recent works have been moving towards exclusively human cells, such as the work by Vatine et al. (2019) where hiPSC derived-astrocytes with neurons and hiPSC derived-brain microvascular endothelial-like cells (BMECs) were included in a sandwich-like design. Interestingly, they demonstrated that the co-culture of BMECs with neurons and astrocytes derived from iPSCs triggers a different gene expression in BMECs compared to when they are co-cultured with primary cells, indicating that the plasticity of ECs depends on their environment. In addition, they employed BMECs derived from Huntington's disease patients in the NVU-oC revealing a significant increase in the permeability of the barrier compared with healthy cells. After that, they used an iPSC model which compromises the thyroid hormone T3 transport. The alteration was introduced using CRISPR/Cas9, and patient-derived cells were utilized. As a result, both NVU-oC models exhibited notably reduced permeability for T3 (Vatine et al., 2019). These results pave the way for a platform for disease modeling and personalized medicine. Similarly, Pediaditakis and collaborators developed Parkinson's disease model. They included primary human pericytes, astrocytes, microglia, hiPSC-derived dopaminergic neurons, and hiPSCs-derived ECs seeded on a sandwich-like design. After the cell seeding, exogenous alpha-synuclein was injected and several aspects of Parkinson's disease were reached after administration, such as accumulation of phosphorylated alpha-synuclein, mitochondrial impairment, neuroinflammation, and barrier function (Pediaditakis et al., 2021). Other applications have been made for NVU-oC such as the stroke model. Lyu et al. (2021) employed a parallel design where human primary astrocytes, pericytes, endothelial cells, human neural progenitor cells, and human microglial cell line (HMC3) were added. They embedded the cells in a 3D matrix from Engelbreth-Holm-Swarm tumor consisting of laminin, collagen, fibronectin, entactin, and heparan sulfate proteoglycans. To induce a stroke, NVU-oC was exposed to serum and glucose deprivation, and hypoxic conditions for 24 hours (Lyu et al., 2021). The proposed ischemic model displayed inflammation and deterioration as expected and neuroprotection and tissue remodeling as *in vivo* stroke models display. Remarkably, some of these works considered the incorporation of shear stress over the NVU by flow conditions which have been extensively studied for its influence on the proper development of the endothelial barrier (Brown et al., 2015; Koo et al., 2018; Vatine et al., 2019; Lyu et al., 2021).

The next generation of NVU-oC will need to consider several aspects to achieve a more complete picture of neurovascular modeling to perform reliable disease study, drug discovery,

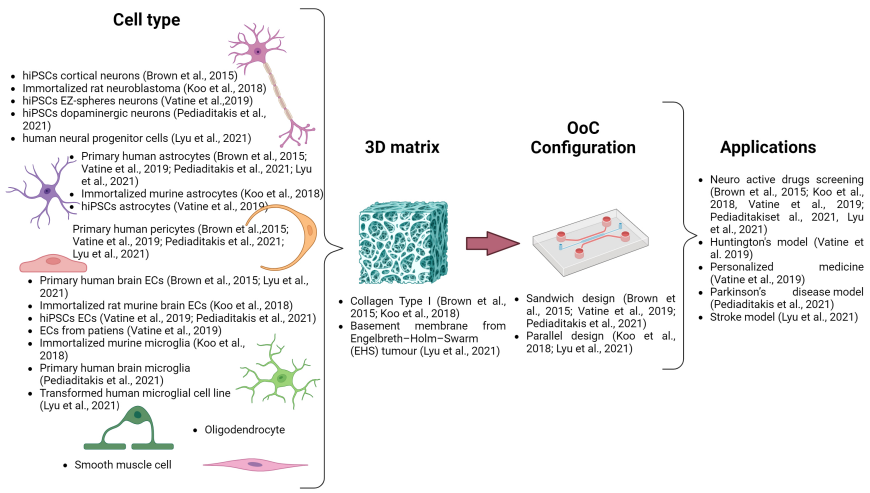

Figure 1 | **Schematic representation of the current developments in NVU-oC.**
Created with BioRender.com. ECs: Endothelial cells; hiPSCs: human induced pluripotent stem cells; NVU: neurovascular unit; OoC: organ-on-a-chip.

and personalized medicine. In this direction, the following points should be considered:

Type of cells: Aforementioned, cells present in NVU play an important role in the physiology and physiopathology of the brain. Therefore, it is important to include some cell types such as neurons in combination with astrocytes, as they provide relevant metabolites for the proper neuronal synapses and function. On the other hand, pericytes produce signaling factors necessary for a tight barrier of ECs in the microvasculature. Additionally, other cell types not included in the platforms reported as oligodendrocytes and microglia are crucial to achieving proper cell functionality. Oligodendrocytes myelinate neurons allowing rapid transmission of electrical signals with neighboring neurons and provide metabolic support. Oligodendrocytes play a role in the initiation and progression of NDDs, thus it is relevant to take them into account (Mathys et al., 2019). Microglia represent the immune system in the central nervous system as it eliminates pathogens, damaged neurons, and plaques. However, it may also have a negative role in the development of NDDs since once activated, it can produce reactive oxygen species and inflammatory factors to induce neurotoxicity (Xu et al., 2021). In addition, SMCs should be considered to mimic large vessels as arteries and arterioles to recapitulate their contraction to regulate the CBF.

Brain regions: The brain has many different regions with specific cellular composition and microenvironment, and therefore functionality. Considering the NDDs intended to study, it is necessary to recapitulate the brain regions involved or even connect several of them. For these purposes, it is important to consider a correct characterization of the specific composition of each region.

Cell source: Cells derived from animals differ from those from humans in terms of phenotype and often miss out on some key functions to recapitulate human physiology properly. Thus, the inclusion of human cells in the chip is necessary. Currently, commercially human cell lines are available along with human embryonic or even mesenchymal stem cells that can differentiate into every cell type of the central nervous system. In addition, human induced pluripotent stem cells (hiPSCs) open the door for patient-derived cell sources allowing personalized medicine on-chip studies. Great advances in the field of stem cells mean there are a multitude of protocols to differentiate them. However, it is important to consider the cell activity and maturity needed for the specific study before choosing them.

The extracellular matrix: The cerebral extracellular matrix contains abundant macromolecules (collagen, enzymes, glycoproteins, and hydroxyapatite) that not only provide a structural support, but also play a role in the activation of biochemical signals and cell behavior. Also, the microenvironment has an important role in brain homeostasis, and its changes could be related to NDDs. To reproduce the extracellular matrix is important to consider especially the proportions of each molecule and their distribution in each brain region. Properties of the container such as the mechanical properties that have relevance in mechano-transduction, cell growth, and cell signaling are also worth consideration. The brain is one of the softest tissues in the body, but it is usually mimicked on glass substrates with high mechanical stiffness that can lead to changes in cell morphology or even bring some cells into the activated state. The matrix closest to the real one is the decellularized brain, where the neurons differentiate better and with a more functional maturation than those cultivated in the 3D collagen matrix.

Cellular arrangement: For a more realistic reproduction of *in vivo* systems, not only the cells used are important, but also their 3D distribution. In the vascularization field, efforts have been made to reduce the diameter of the vasculature and to obtain a tubular shape of the vessels using strategies as vasculogenic processes on the chip. For neural cultures, microcontact and 3D patterns on surfaces have been used to achieve the right directionality of neurons on the chip.

High throughput: Considering that one of the main commercial applications of this type of platform is drug discovery and screening, it is necessary to adapt the manufacture of this type of platform to process multiple compounds in a cost-effective manner. There are reported examples of high throughput platforms for BBB or even more complex multi-organ oC platforms (Azizgolshani et al., 2021), but not yet for NVU-oC and many other OoCs.

Integrated monitoring systems: The primary method of characterizing OoC is by immunostaining analyzed with microscopy. If this technology is to have a real commercial use, it will be necessary to integrate real-time, automated, non-invasive, and reusable monitoring systems into the chip such as electronic/electrochemical sensors, some examples in Mir et al. (2022).

In conclusion, OoC technology allows incorporating important features such as different configuration, cell types, cell-cell and cell-matrix interactions, biomaterial stiffness, and shear stress for a more reliable study of human physiology *in vitro*. However, most of the current NVU-oC models are still generic, lacking some relevant reproducing of specific brain regions. Despite these limitations, continuous progress is being conducted with particular emphasis on the integration of monitoring systems for automated, non-invasive, and real-time recording.

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