Immunocompetent brain organoids—microglia enter the stage

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TOPICAL REVIEW

Immunocompetent brain organoids—microglia enter the stage

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Abstract

Microglia, the immune cells of the brain, are a focus of studies in neurodegenerative diseases. Similarly, research about induced pluripotent stem cell (iPSC)-derived whole brain and region-specific organoids is increasing. In organoids, the complexity of the culture systems increases, mimicking better the actual scenario in the human brain. Furthermore, animal models do not always recapitulate human neurodegeneration, and they imply more ethical concerns compared to organoid systems. Recently the integration of iPSC-derived microglia into brain organoids has been achieved, and on-chip technologies have been focusing on microglia interaction with neural cells. In this review, we discuss the achievements on integrating microglia into brain organoids. We study the cell organization, ultrastructure and cell signalling of microglia with respect to other cell types in organoids as well as their functionality in the system. A particular focus here is on the interaction with the midbrain and dopaminergic systems. Finally, we discuss the achievements until now concerning neuroinflammation and disease modelling, and the possible therapeutic approaches targeting microglia and neuroinflammation in 3D systems.

1. Introduction

Microglia are the innate immune cells in the brain, which ensure the correct physiology of neurons and other cell types. They inspect their environment thanks to their motile processes and clear the brain parenchyma from cell debris, tissue components and metabolic waste products (Nimmerjahn *et al* 2005). They recognize apoptotic neurons through complex surface signalling (Witting *et al* 2000). During development, microglia regulate cell death and proliferation in the neurogenic niche via phagocytosis. They have, thus, a crucial role in maintaining a clean environment, reducing stress and toxicity to neural cells.

Microglia physically interact with neurons through contacts between microglial processes and neuronal projections and soma, where they establish signalling pathways through purinergic receptors (Cserép *et al* 2020). There is a bidirectional communication between microglia and neural precursors in the developing brain (Arnò *et al* 2014). Recent studies showed that, in mice deficient for the P2RY12 and MERTK/Axl phagocytosis pathways, neurogenesis was disrupted. Therefore, the phagocytosis ability of microglia is important not only to clear cell debris and waste products, but also for neurogenesis in the developing brain (Diaz-Aparicio *et al* 2020).

Microglia have self-renewal ability, which ensures their preservation and long-lasting presence in the brain. Studies showed the re-arrangement of microglia in the post-natal brain, and the ability to re-populate the brain regions via self-renewal after a substantial cell number loss (Bennett *et al* 2016, Eyo *et al* 2018). Analysis in the human brain cortex showed that microglia have a yearly turnover of 28%. Most of the microglia cells will have a relatively short lifespan; on average, they have an age of 4.2 years. Despite the fact that the majority of microglia are renewed, a small subset—constituted by less than 4% of the total cells—can live up to 20 years (Réu *et al* 2017).

Microglia, unlike macroglia and neurons, do not have a neuro-ectodermal origin, but arise from mesodermal extra-embryonic tissue and invade the developing brain at early embryonic stages. After

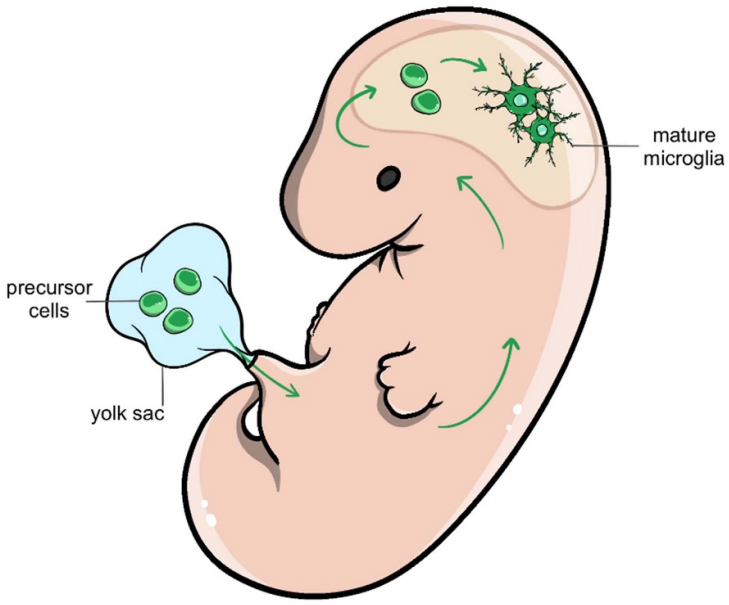

Figure 1. Ontogeny of human microglia. Microglia precursors develop in a mesodermal extraembryonic tissue, called the hemogenic endothelium, located in the yolk sac. These precursors migrate through vasculature to the developing brain, and enter the tissue through the telencephalon around gestational week 4.5. Once in the central nervous system's (CNS) parenchyma, they colonize the different regions of the developing brain (Ginhoux *et al* 2010, 2013, Menassa and Gomez-Nicola 2018).

gastrulation, extra-embryonic mesoderm develops in the yolk sac (Lacaud and Kouskoff 2017) where, during the third week of human gestational age, primitive haematopoiesis begins. Stem cells have a myeloid and erythroid commitment (Ginhoux *et al* 2013) and they will give rise to primitive erythrocytes, megakaryocytes and macrophages. Later on, the rest of the erythroid-myeloid lineage, as well as lymphoid cells, will develop during definitive haematopoiesis in the yolk sac, foetal embryo and in the bone marrow, being the latest persistent during post-natal age (Tavian and Péault 2005, Lacaud and Kouskoff 2017). Myeloid stem cells—expressing IBA1, CD68, CD45 and MHC-II—will develop into macrophage precursor through the expression of transcription factors such as PU.1 (Chan *et al* 2007).

Around week 4.5 of gestational age in humans, these precursor cells migrate from the yolk sac to the developing brain through blood vessels (Ginhoux *et al* 2010), and enter through the telencephalon in the forebrain (Ginhoux *et al* 2013, Menassa and Gomez-Nicola 2018, figure 1). After this, they colonize the developing brain regions, reaching the midbrain region by week 22 of human gestation (Menassa and Gomez-Nicola 2018). This invasion process occurs prior to the blood-brain barrier formation and, therefore, the developing central nervous system is easily accessible by microglia precursor cells. Once microglia enter the central nervous system, they subsequently distribute through different developing regions. Microglia expand until they reach different proportions depending on the brain region, which will stay stable during adulthood. Immunohistochemistry studies have shown that, in adult age, microglia represent around 10% of the cells in the *substantia nigra* of the midbrain (Mittelbronn *et al* 2001). The formation and closure of the blood-brain barrier shortly after the microglia precursor invasion makes the access to the central nervous system by external cells difficult and rare. However, in rare occasions, circulating blood monocytes can enter the central nervous system through the blood-brain barrier following infection or inflammation and differentiate into resident microglia (Ginhoux *et al* 2013).

Because of the highly interactive network in which microglia participate in the healthy and diseased brain, their presence in advanced cell culture systems is essential for an accurate recapitulation of the human brain development, physiology and pathology. In this review, we discuss the recent advances in the field of human microglia in cell culture models, focusing on induced pluripotent stem cell (iPSC)-derived systems and brain organoids.

2. Historical perspectives on microglia modelling

In the 1910s, Pío del Río Hortega studied in depth glial cells, identifying microglia for the first time and discussing their similarities with the 'third element', described by Santiago Ramón y Cajal. By using human

Table 1. Immortalized human microglia cell lines. Cell lines used over the last decades, origin and immortalization method.

Cell line	Donor age	Tissue	Immortalization technique	Reference/First use
HMC3 (also named CHME-5, CHME3, C13-NJ)	Embryonic	Spinal cord and brain cortex	Viral transduction with SV40 Large T antigen	Janabi *et al* (1995)
HMO6	Embryonic	Telencephalon	Viral transduction with *v-myc* oncogene	Nagai *et al* (2001)
Huμglia	Adult	Brain cortex	Viral transduction with SV40 Large T antigen	Garcia-Mesa *et al* (2017)
SV40	Adult	Brain	Viral transduction with SV40 Large T antigen	Chiavari *et al* (2019)/ Commercial line

post-mortem brain material—and confirming his results with other vertebrate material—he described in detail some of the morphological and physiological features of microglia. He used human tissue and obtained 20 μm cryosections, which were shortly treated with formalin bromide, stained with ammonia-silver carbonate and toned with gold chloride. The short treatment with formalin bromide allowed his team to specifically stain microglia, and distinguish them from what he called 'neuroglia' (astrocytes). Thanks to this technique, Hortega investigated microglial morphology, their distribution in the brain and the different morphological types of microglia (Río Hortega 1919a). He also studied the morphological changes of microglia in pathological conditions such as infection, describing hypertrophy and hyperplasia of microglia in inflammatory processes. He discussed the migration of microglia cells throughout the brain parenchyma, and observed the morphological changes of microglia and their accumulation around blood vessels in neurodegeneration (Río Hortega 1919b).

Furthermore, he discussed the mesodermal origin of microglia in contrast with the astrocyte ectodermal ontogeny. He described the morphological differences between 'neuroglia' (astrocytes) and microglia; in particular, the differences in the centrosome and Golgi apparatus, as well as the fact that specific staining for one of the cell types would not stain the other. Furthermore, he stated that, while astrocytes preserve their shape during their lifespan, microglia change their morphology and migrate throughout the brain. The only observed similarity between these cell types was their 'star-shape'. He used these evidences to support the hypotheses of peer-researchers (such as Marinesco and his hypothesis of microglia having a mesenchymal origin). He clarified that further assays should be done to confirm their ontogeny, but concluded his speculations by confirming a possible mesodermal origin of microglia. (Río Hortega 1919c).

Finally, the mobility and phagocytic activity of microglia was assessed and discussed. By generating a lesion in the cortex of newborn cats and sacrificing them at different time points, Hortega and his team observed an increase of microglia abundance around the wound throughout time. They also noticed that, over time, there was an increase of red blood cells detected in the cell bodies of microglia around the wound, indicating that these cells had phagocytic capacity. Eventually Hortega described microglia as 'scavenger cells', as well as macrophages that collect, store and degrade cell debris (Río Hortega 1919d, Sierra *et al* 2016).

After these investigations, research in microglia was centred on their morphology and their phagocytosis ability, mostly in gliomas (Penfied 1925, Kurobane 1950, Imamura 1954). Those studies were of great value for further investigations about microglia, re-taken in the 1960s, where the first insights into synaptic remodelling were assessed and discussed (Blinzinger and Kreutzberg 1968). In these studies, the samples used were animal material and post-mortem human brain tumour tissue. Later on, murine (Aizawa *et al* 1991, Walton *et al* 2000, Adami *et al* 2004) and human (Cross and Nicola Woodroofe 1999, Fillebeen *et al* 2001, Nagai *et al* 2005) cell lines were developed and applied for the study of microglia *in vitro.*

The development of human cell lines overcame the low accessibility of post-mortem samples, since human lines could be easily expanded and shared within the scientific community. Human microglia cell lines have been established over the past decades, obtained by immortalization of adult (Garcia-Mesa *et al* 2017, Chiavari *et al* 2019) or embryonic microglia (Janabi *et al* 1995, Nagai *et al* 2001, table 1, figure 2). These lines are of high value, since they have allowed for studies of human inflammatory processes *in vitro.* However, the variability between individuals and the many disorders in which microglia are involved make these models not always sufficient. With the discovery of iPSCs and cell reprogramming, a door opened towards the study of microglia in a personalized medicine perspective, and for all kinds of neurological disorders.

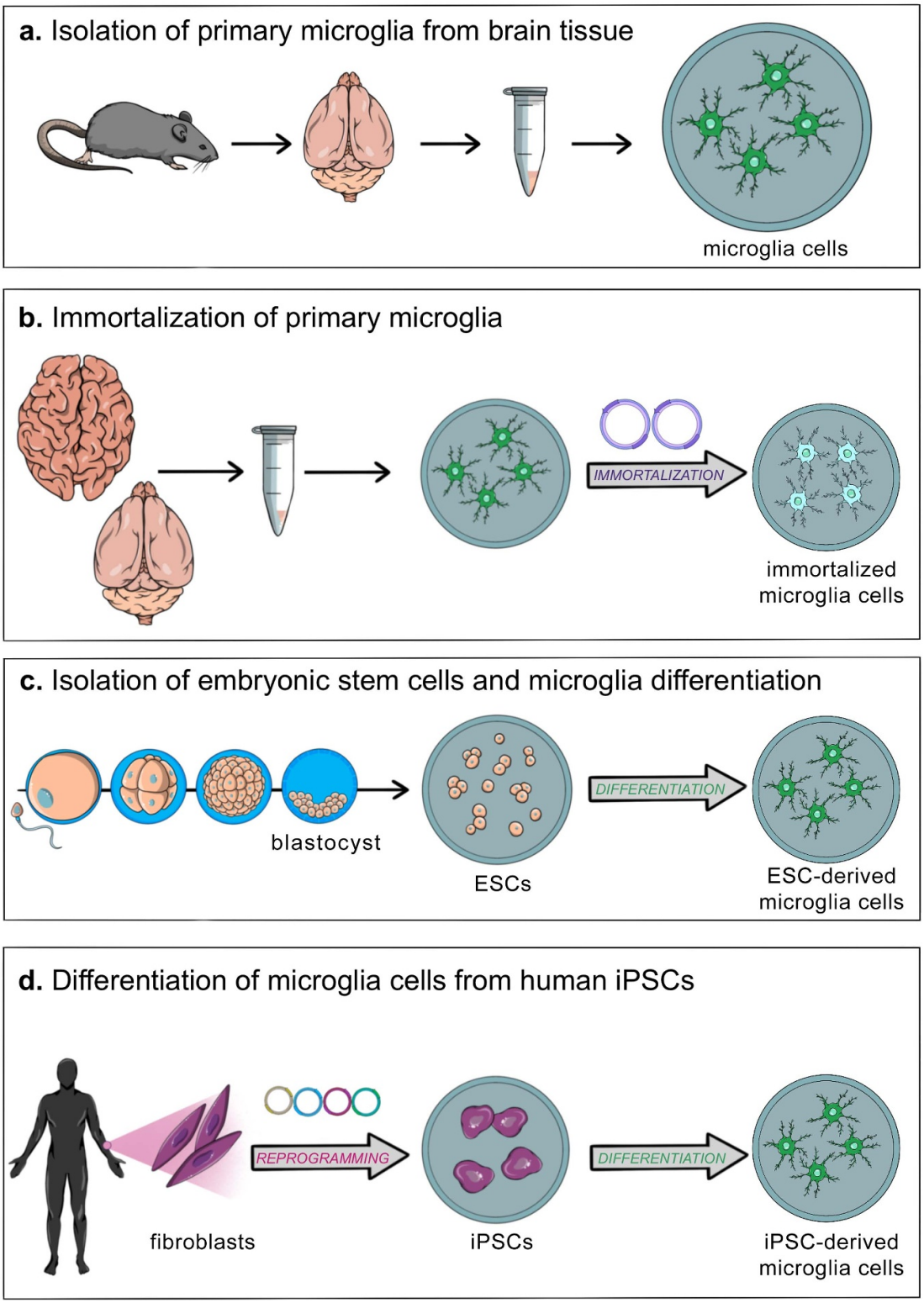

Figure 2. Methods to obtain microglia for 2D cell culture. (a) Murine (and, in more rare cases, human) microglia can be isolated from brain tissue and brought in culture. (b) Microglia isolated from murine or human brain tissue can be immortalized, obtaining stable microglia cell lines that can be used for prolonged time periods. (c) Embryonic stem cells (ESCs) can be isolated from human blastocysts and differentiated into microglia by exposing the cells to specific culture factors. (d) Induced pluripotent stem cells (iPSCs) can be reprogrammed from human fibroblasts (or other cell types) and differentiated into microglia in culture.

3. From iPSCs to organoids: current approaches to study microglia interactions with neural cells

The development of PSC generation in 2006 by (2006) represented the start of modern cell culture, which awarded Shinya Yamanaka with the Nobel Prize in physiology and medicine in 2012. They discovered that

Table 2. Human iPSC-derived microglia derivation protocols developed over the last years.

Reference	Source of iPSCs	Derivation technique	Total protocol duration
Muffat *et al* (2016)	Fibroblasts	YS-EB formation, positive selection of single cells and microglia maturation	∼74 days
Abud *et al* (2017)	Fibroblasts/Adult PBMCs	Differentiation into hematopoietic progenitors and microglia differentiation	∼39 days
Pandya *et al* (2017)	$CD34^+$ embryonic/adult cells	Myeloid differentiation, $CD34^+/CD43^+$ cell selection, differentiation into astrocytes/microglia and MACS sorting for $CD11b^+$ microglia cells	∼29 days
Douvaras *et al* (2017)	Fibroblasts	Progenitor derivation, $CD14^+$ cell isolation and microglia differentiation	∼45–60 days
Haenseler *et al* (2017)	Fibroblasts	Macrophage precursor derivation via YS-EB formation (van Wilgenburg *et al* 2013) and microglia differentiation/co-culture with neurons	∼42 days
Takata *et al* (2017)	Fibroblasts	Induction of hemangioblast-like cell formation, macrophage derivation, $CD45^+CD11b^+CD163^+CD14^+CX3CR1^+$ FACS sorting and co-culture with neurons	∼30 days

human adult fibroblasts can be re-programmed into stem cells, called iPSCs via overexpression of Oct3/4, Sox2, Klf4 and c-Myc. iPSCs can then be differentiated into most cell types in the human body. Their findings opened the door to a different approach of cell culture studies, overcoming the limitations of human cell availability and making a step forward into personalized medicine (Takahashi *et al* 2007).

3.1. iPSC-derived microglia

The iPSC technology allowed researchers to study cellular and molecular aspects of brain physiology and disease, overcoming the limitation of sample availability and difficulties to obtain human brain biopsies. Differentiation of iPSCs into microglia has been achieved in multiple studies (Haenseler *et al* 2017, Panagiotakopoulou *et al* 2020, Badanjak *et al* 2021, table 2 and figure 2). This opened the door to an accurate way to study human neuro-immune processes in health and disease, with a personalized medicine approach. Generating iPSC-microglia often represents an advantage compared with human embryonic PSC and cell line-derived cells, since they preserve the genetic information from the donor. Furthermore, medical practitioners can obtain skin biopsies from their patients—a relatively easy and slightly invasive procedure—that can be used for re-programming into iPSCs. This translates into a high amount of human cell lines representing many disorders and different individuals, leading to extensive, detailed and well-represented research studies.

3.2. Microglia in 2D co-culture systems

Interactions between microglia and other brain cells have been studied *in vitro* via 2D co-culture. Unlike mono-cellular 2D culture systems, co-cultures allow for the understanding of contacts and molecular interactions between cell types. Researchers have applied co-culture systems of human iPSC-derived cortical neurons with iPSC-microglia to study inflammatory response (figure 3, Haenseler *et al* 2017). In Haenseler *et al* (2017), the transcriptomic identity of iPSC-microglia was compared to human blood monocytes and foetal microglia, showing a higher similarity to foetal microglia. These results indicated that the differentiation of microglia from iPSCs by using a specific protocol and medium composition can recapitulate their embryonic origin.

Microglia in co-culture with cortical neurons displayed a ramified morphology, had phagocytosis ability and responded to lipopolysaccharide (LPS) stimulation by producing cytokines and chemokines (Haenseler *et al* 2017). Therefore, iPSC-microglia were functional in terms of response to stimuli and cell signalling ability. Similarly, in Lopez-Lengowski *et al* (2021), iPSC-microglia and neurons were co-cultured and the neuronal dendritic spine amount was quantified. In that study, the spine count and length, as well as the neurite length of cortical neurons co-cultured with microglia did not differ from the values from neurons in monoculture (Lopez-Lengowski *et al* 2021), indicating that microglia did not interfere in the development of dendritic spines in 2D.

Studies where primary (Goshi *et al* 2020) or iPSC-derived (Haenseler *et al* 2017) cortical neurons were successfully co-cultured with microglia indicate that these two cell types can be co-cultured with the same

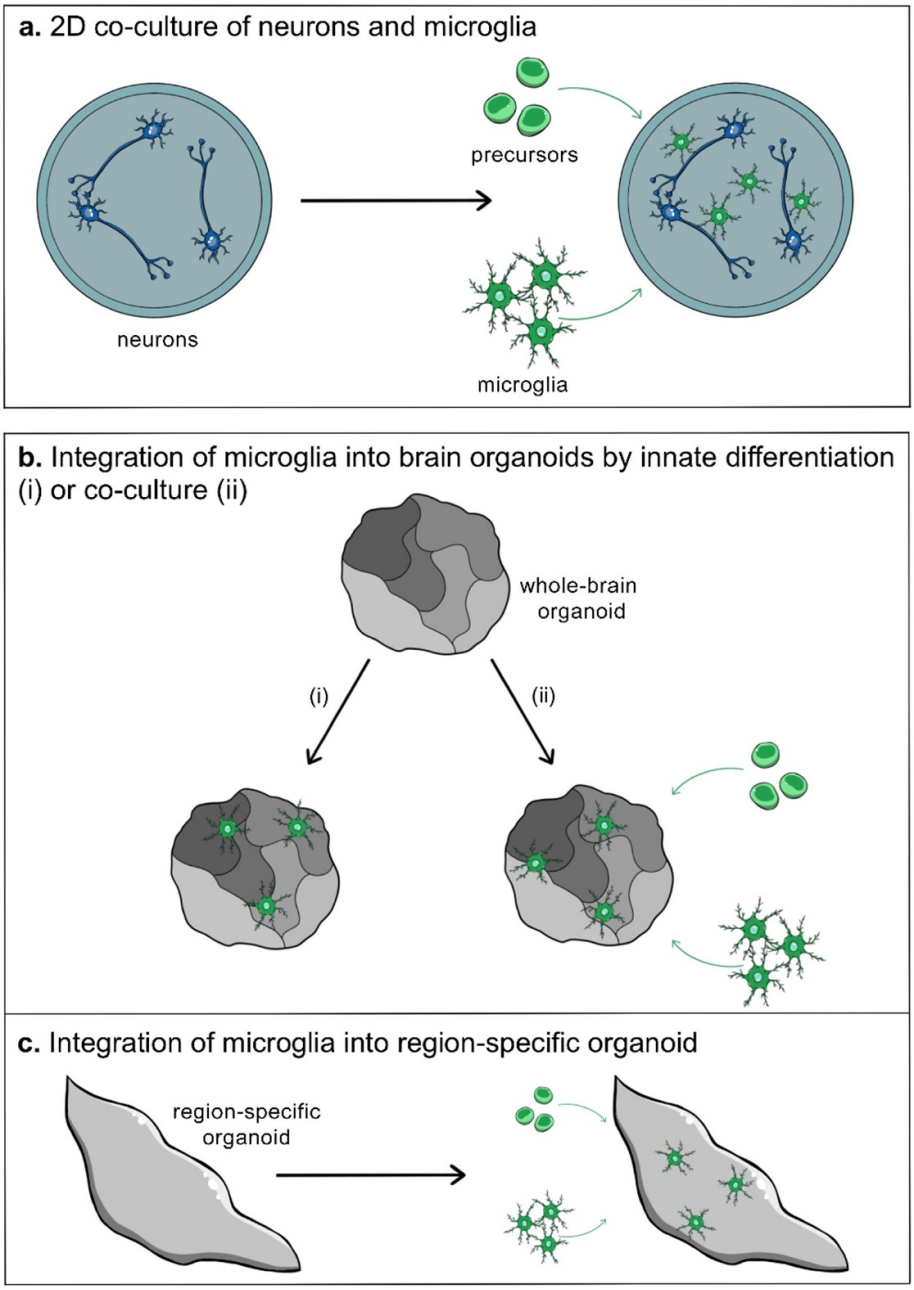

Figure 3. Studying microglia interactions with neural cells by 2D and 3D co-culture. (a) Microglia precursors or differentiated cells can be co-cultured in 2D with neurons. (b) (i) In order to generate microglia-containing organoids, an innate induction can be performed in whole-brain organoids (Ormel *et al* 2018, Gabriel *et al* 2021, Cakir *et al* 2022). (ii) Microglia precursors or differentiated cells can be integrated into whole-brain organoids by 3D co-culture (Muffat *et al* 2016, Abud *et al* 2017, Popova *et al* 2021, Fagerlund *et al* 2021). (c) Microglia can be integrated into region-specific organoids by 3D co-culture with precursor cells or differentiated microglia (Bejoy *et al* 2019, Sabate-Soler *et al* 2022).

culture medium without compromising their viability or differentiation capacity. However, there are fewer studies showing successful co-culture between iPSC-derived microglia and dopaminergic neurons.

Interestingly, co-culture of primary midbrain-specific neurons with primary microglia has been achieved in order to study neuroinflammation in Parkinson's disease (PD) (Tu *et al* 2019). Moreover, an indirect co-culture method of primary microglia with iPSC-derived neural precursors demonstrated the capacity of microglia to promote tyrosine hydroxylase-positive cell differentiation (Schmidt *et al* 2021). Nonetheless, no iPSC derived microglia were used in that study, but human and mouse primary cells. Thus, microglia were already in a differentiated state and were cultured in basal medium without small molecules that induce microglia differentiation. Furthermore, the contact between microglia and neurons was established through inserts and for a relatively short time period of five days (Schmidt *et al* 2021). While that work is highly valuable to understand the effects of microglia in dopaminergic differentiation *in vitro*, it does not demonstrate the long-term viability and identity of microglia and dopaminergic neurons in co-culture.

The lack of studies where iPSC derived dopaminergic neurons are co-cultured with iPSC-derived microglia—which implies inducing differentiation of both cell types—could be due to the technical difficulties in engineering a culture medium that induces both dopaminergic neuron and microglia differentiation. Co-culturing neurons and microglia derived from iPSCs represents an advantage with respect to other systems, since both cell types could be derived from the same individual (the same skin biopsy). This allows the study of phenotypes and interactions between cell types with the same genetic background, which can be highly advantageous to assess disease phenotypes.

Despite the fact that 2D co-culture systems gave more information to researchers in comparison to monocultures, these systems often fail to recapitulate the 3D microenvironment of human tissue (Moysidou and Owens 2021).

3.3. Integration into whole brain and region-specific organoids

With the development of human stem cell-derived organoid systems, the limitations of classical cell culture, to mimic complex 3D tissue microenvironment were largely overcome. Starting from PSCs, this initial population of cells differentiates into to multiple cell types in 3D, giving rise to spheroids and organoids with different types of cells that interact with each other in a microenvironment that resembles the one in human tissue and organs. The first 3D tissue culture models were composed by tissue-specific cell types (often co-cultured and layered) embedded in an extracellular matrix gel, which allowed for the study of (patho) physiological processes (Gjorevski *et al* 2014, Lancaster and Knoblich 2014, Caddeo *et al* 2017).

3.3.1. Brain organoids

Organoid models have a cellular heterogeneity, with multiple cell types that connect and communicate with each other in a tissue-like manner. Brain organoids have been developed during the last decade to study neural development and neurological disorders (Lancaster *et al* 2013, Lindborg *et al* 2016, Gomez-Giro *et al* 2019).

Because of the mesodermal origin of microglia, they do not tend to innately differentiate within the neuroectodermal tissue of brain organoids. However, this innate specification depends on the culture method of the organoids. Un-guided differentiation approaches lead to self-organization of cellular structures from different lineages. The culture medium composition and concentrations focus on preserving brain cell identity and survival; therefore, these approaches could lead to an innate microglia differentiation. A human ESC-derived brain organoid model showed invasion of microglia cells upon transplantation into the mouse brain (Mansour *et al* 2018). While this protocol leads to human brain organoids with microglia, they retain the limitation of transplantation and the fact that microglia cells are not human. Interestingly, the induction of an innate microglia differentiation within iPSC-derived human brain organoids has been achieved via overexpression of the microglial transcription factor PU1 (Cakir *et al* 2022). In contrast, previous work has described an innate differentiation of microglia in human iPSC-derived cerebral organoids (Ormel *et al* 2018) and optic vesicle-containing brain organoids (Gabriel *et al* 2021) without any gene overexpression. In Ormel *et al* (2018), researchers compared the transcriptomic identity of microglia in brain organoids with human adult and foetal microglia. They showed that microglia in organoids correlated with adult microglia, while microglia 2D cultures correlated with foetal human microglia. These gene expression-based results were validated by functional studies, and suggest that microglia maturation and functionality *in vitro* increases in 3D with respect to 2D. Nonetheless, microglia differentiation does not always occur innately in iPSC-derived cerebral organoids. In multiple unguided differentiation approaches, microglia had to be externally assembled since they did not develop innately (figure 3, Muffat *et al* 2016, Abud *et al* 2017, Popova *et al* 2021, Fagerlund *et al* 2021).

3.3.2. Region-specific organoids

To specifically target brain processes in discrete brain regions, such as forebrain and midbrain, region-specific brain organoids have been engineered. Some examples are forebrain (Birey *et al* 2017), cortical (Qian *et al* 2016) and midbrain organoids (Jo *et al* 2016, Monzel *et al* 2017). In order to obtain region-specific organoids, the applied cell culture approach is a guided differentiation, where small molecules are applied at specific concentrations and time exposures, leading to specificity and differentiation of the tissue. Guided cell culture differentiation often implies a pre-patterned initial cell population (Jacob *et al* 2021), for instance, neural floor plate progenitor cells in the case of midbrain organoids (Smits *et al* 2019). Because of the high specificity of the target identity in guided differentiation approaches, there is a very low chance of innate differentiation of mesoderm-derived compartments. That is why these cell types—like microglia—have to be generated in an additional culture and added later.

Prenatal human microglia have been isolated and integrated to iPSC-derived cortical organoids (Popova *et al* 2021). In this study, it is shown that microglia isolation from human foetal brain lead to a decrease of

the expression of homeostatic genes. This decrease improved upon transplantation into the mouse brain and, interestingly, also upon integration into human cortical organoids. The restoration of the microglial cytokine signature was fully achieved upon co-culture with human-derived organoids, but to a lesser extend upon transplantation into mice. This was due to the fact that certain cytokines that can be found in humans of young age are only produced by aged mice. This was the case of the cytokines interleukin 1 beta (IL-1β), chemokine (C-C motif) ligand 3 (CCL3) and chemokine (C-C motif) ligand 4 (CCL4), which exemplified the importance of human material for certain inflammatory studies, and the differences with respect to animal models (Popova *et al* 2021).

The limitation of this co-culture approach is that it implies the isolation of microglia from human brain, which can be of low accessibility. Several articles followed an approach where microglia were derived from human iPSCs and incorporated to the organoids by co-culture at a certain time point during organoid culture (figure 3). This has been done by integrating fully differentiated microglia or cells in a precursor state, followed by a microglia differentiation within organoids. In Song *et al* (2019), iPSC-derived human microglia were successfully co-cultured with dorsal or ventral cortical organoids. Other protocols have assessed a successful integration of iPSC-microglia into cortical spheroids (Bejoy *et al* 2019). In Xu *et al* (2021), iPSC-microglia were co-cultured with brain organoids, but also dorsal and ventral forebrain-specific organoids. An advantage of these approaches is that the ratio of microglia in organoids can be easily controlled, since the amount of microglia integrated can always be modulated. This is of high value, taking into consideration the different proportions of microglia throughout brain regions.

Until recently, no 3D studies showed microglial presence in midbrain-specific organoids. However, iPSC-microglia have been successfully integrated into human midbrain-specific organoids (Sabate-Soler *et al* 2022) via co-culture with macrophage precursors using a specifically designed co-culture medium (figure 3). In that approach, cells were integrated in a precursor state and differentiated into microglia in 3D. It was shown that dopaminergic neurotrophic factors were compromising microglia survival in 2D and 3D, and the microglia medium was not enhancing dopaminergic differentiation in 3D (Sabate-Soler *et al* 2022). The fact that a specific medium had to be developed could explain why there is so far only one study of midbrain-specific organoids with microglia (Sabate-Soler *et al* 2022).

3.3.3. Microglia functionality and identity within organoids

Over the past few years, researchers have successfully integrated iPSC-microglia into brain organoids, which represents a great advance in terms of cellular heterogeneity and complexity of these systems. However, the question whether iPSC-derived microglia have a primary microglia identity, or they should rather be called 'microglia-like cells' remains for these 3D models. In this section we discuss the functionality of iPSC-derived microglia in 3D models, and describe the similarities and differences with respect to *in vivo* and primary microglia.

One of the functional features of microglia is the ability to produce cytokines in response to inflammatory stimuli (Giulian *et al* 1986, Giulian 1987, Sawada *et al* 1989, 1990). iPSC-derived microglia have been shown to have cell signalling abilities when innately differentiated in brain organoids, since they increased their release and expression of cytokines—such as IL-6 and TNFα—after LPS stimulation. Furthermore, protocols where cells have been externally incorporated have also led to functional microglia in organoids. For example, microglia co-cultured with tubular forebrain organoids, generated in a hollow mesh scaffold responded to pro-inflammatory stimuli in terms of inflammasome activation and cytokine production (Ao *et al* 2021). In that work, microglia activation was assessed, as well as their branching complexity, cytokine secretion levels and NLRP3 inflammasome activation (Ao *et al* 2021). In Song *et al* (2019), microglia within dorsal and ventral cortical organoids responded to pro-inflammatory stimuli, showing that they are functional within the co-cultured system. Microglia integrated within midbrain-specific organoids secreted cytokines and chemokines, and expressed phagocytosis and inflammation-related genes. Amongst them, pro-inflammatory cytokines such as IL-6 and TNFα, and anti-inflammatory cytokines, such as IL-10 were produced by microglia in organoids, indicating that they could induce different types of responses in neural cells present in the system (Sabate-Soler *et al* 2022).

Microglia within organoids have been shown to modulate stress levels and cell viability. In Popova *et al* (2021), cortical organoid with microglia showed less DNA breaks than organoids without microglia. Midbrain organoids containing microglia have also shown lower amounts of dead cells, as well as a down-regulation of oxidative stress and hypoxia-related genes in comparison to organoids without microglia. Interestingly, midbrain organoids with microglia showed a smaller size than organoids without microglia (Sabate-Soler *et al* 2022). These observations demonstrate that microglia may be functional within midbrain organoids, since microglia in the brain clear metabolic waste products and phagocyte apoptotic neurons (Witting *et al* 2000). However, further analysis assessing functional mechanisms of microglia in 3D will be of high value to confirm these observations. For example, molecular assays assessing cell viability and

oxidative stress could be performed to validate that microglia phagocytose metabolic waste products and debris, reducing stress and increasing viability in organoids. Furthermore, assessing hypoxia in organoids with microglia would allow researchers to understand whether phagocytosis of cell debris and small organoid size leads to a better oxygen diffusion throughout the organoid tissue.

In the brain, microglia interact with neural cell types and affect synaptic functions as well as perform synapse pruning, (Wake *et al* 2009, Tremblay 2012). 2D co-culture systems of primary mouse microglia and neurons have been used to study synapse pruning and spine elimination *in vitro* (Cheadle *et al* 2020, Scott-Hewitt *et al* 2020). Synaptic pruning has also been assessed in 3D systems containing microglia, mainly by immunodetection of synaptic and microglial markers, and studying their co-localization via high resolution microscopy. In Xu *et al* (2021), they showed a co-staining of the microglial markers IBA1 and CD68, co-localizing with the post-synaptic marker PSD95 in iPSC-derived brain organoids. A similar approach was used in Fagerlund *et al* (2021) and Popova *et al* (2021), where co-localization of synaptic markers—such as PSD95 and Synaptophysin—and microglia markers—like IBA1 and CD68—was observed in cerebral and cortical organoids, respectively. In midbrain-specific organoids, a down-regulation of synaptic gene markers, as well as lower protein levels of the pre-synaptic vesicle marker VAMP2, were observed in the presence of microglia compared to organoids without microglia. This was accompanied by an up-regulation of synaptogenesis and action potential-related genes, suggesting that microglia within midbrain organoids could be phagocytosing inactive synapses, improving the functionality of the remaining ones (Sabate-Soler *et al* 2022). Further assays exploring synapse pruning in midbrain organoids will be important in order to confirm the downregulation of synaptic genes observed. An example is a high-resolution microscopy approach to detect tagged synaptic proteins within microglia cell bodies, as shown in other articles. Treatment of microglia-containing organoids with fluorescently labelled synaptosomes would be another possible approach to assess synapse pruning in 3D.

Considering that synapse pruning is associated to an efficient synaptic transmission and neuronal functionality, some of these works assess electrophysiological properties in organoids with microglia. In Fagerlund *et al* (2021), patch clamp recordings with cerebral organoids with or without microglia were performed. Higher densities of potassium and sodium currents occurred were observed in the presence of microglia. Improved electrophysiological properties have also been observed in cortical organoids with microglia, where a higher frequency of oscillatory bursts was observed in comparison to organoids without microglia. In midbrain organoids with microglia, patch clamp measurements showed a lower action potential threshold in organoids with microglia, and a lower inter-spike interval were recorded via multi-electrode arrays compared to organoids without microglia (Sabate-Soler *et al* 2022), showing an increased spontaneous activity. These results support the observations and hypotheses that microglia within organoids perform synaptic pruning, increasing the electrophysiological properties and synaptic transmission of the remaining synapses. Future efforts could focus on assessing whether the improved synaptic functionality is accompanied by higher neuronal maturation in organoids with microglia via specific marker expression and morphological features such as spine formation.

A limitation of iPSC-derived microglia in 2D and 3D is the amoeboid-like microglia morphology within organoids. The fact that iPSC-derived microglia display an amoeboid morphology that resemble human microglia upon injury or disease has been previously discussed (Hasselmann and Blurton-Jones 2020, Sabate-Soler *et al* 2022). The morphological complexity of 2D iPSC-derived microglia tends to increase in 3D systems, but it often needs molecular queues from the CNS upon transplantation into animal models to display a comparable morphology to the one seen *in vivo* (Hasselmann and Blurton-Jones 2020). Indeed, human iPSC-derived microglia cells increased their branching and morphological complexity upon transplantation into mice brain (Hasselmann *et al* 2019, Svoboda *et al* 2019, Xu *et al* 2020).

The innate differentiation of microglia within cerebral organoids seemed to display a more ramified morphology (Ormel *et al* 2018), however microglia do not reach the same appearance as they do in studies where a transplantation into mouse brain was done. The fact that iPSC-derived microglia do not reach a complex morphology could be explained by the exposure to stress linked to technical manipulations of isolated iPSC-derived microglia, which could induce morphology changes in the cells. More importantly, the molecular queues from the CNS are absent in these derivation approaches. This hypothesis is supported by a study where, shortly after isolation from the CNS, primary microglia lost their differentiated gene identity, which was restored upon re-transplantation into the mouse brain (Bohlen *et al* 2017). Furthermore, upon transplantation into mice brain, the gene expression profile of those cells is much similar to primary microglia, in comparison with isolated iPSC-derived microglia. Visually, it seems that microglia in organoids do not reach such complex branching features with respect to transplanted or *in vivo* microglia, which suggests that the organoid tissue does not provide with the necessary support for microglia to branch and, possibly, to reach the same gene identity as in the human brain. Future efforts could focus on bringing microglia in midbrain organoids closer to the physiological state in the human brain by, for instance,

reducing the high levels of stress in the system. For this purpose, a possible option could be obtaining a less compact tissue and therefore enhance passive diffusion of oxygen and nutrients to the core. Organoid perfusion through tubular structures or scaffolds could also be a suitable option to promote oxygen and nutrient flow throughout the organoid. A different approach might focus on supplementing the culture media with antioxidants to try to reduce oxidative stress in the system. Furthermore, an organoid model with features closer to the actual CNS (e.g. containing blood vessels, cerebrospinal fluid, different inter-connected regions with mature neurons, etc) may lead to a more accurate microglial identity.

Overall, iPSC-derived microglia in organoids recapitulate many microglial functions *in vivo*, such as cell signalling via cytokine production, process motility, synaptic pruning and electrophysiology. While this is highly advantageous to study neuroinflammation in 3D, the limitation of low morphological complexity indicates that microglia in organoids may not have an accurate identity with respect to microglia *in vivo*. A next generation of brain organoids, where microglia have a similar morphology and identity to primary microglia, would lead to more accurate modelling of inflammatory processes in the healthy and diseased brain.

4. Modelling neuroinflammation in 3D

One of the most known processes that accompany neurodegeneration is neuroinflammation, where immune brain cells develop an inflammatory response to injury or infection. Microglia belong to the group of innate immune system cells, which respond rapidly to injury through a short-term response. Microglia, together with astrocytes, are the main cell types responsible for neuroinflammation (Troncoso-Escudero *et al* 2018). Necrosis signals can be recognized by microglia and astrocyte receptors, e.g. Toll-like receptors and nucleotide oligomerization domain receptors. Furthermore, endogenous molecules like aggregated α-synuclein can be recognized by microglia and astrocytes and trigger an immune response (Roodveldt *et al* 2010, Troncoso-Escudero *et al* 2018). Microglial activation upon neuronal damage and protein accumulation leads to a morphological change, where microglia processes retract and cells acquire an amoeboid morphology. These morphology changes are accompanied by a molecular response cascade, including the production of cytokines and chemokines, activation of inflammatory pathways (such as nuclear factor kappa B pathway) and oxidative stress (McGeer *et al* 1988, Członkowska *et al* 1996, Hirsch and Hunot 2009). In neurodegenerative disorders, such as PD, microglia activate upon recognition of molecules, like α-synuclein or necrosis signals from neurons. This leads to a response cascade where microglia release pro-inflammatory molecules and neurotoxic factors that contribute to neuronal damage. This further supports microglial activation, creating a pro-inflammatory positive-feedback loop called reactive microgliosis (Castaño *et al* 1998, Block *et al* 2007, Tansey and Goldberg 2010, Couch *et al* 2011).

Functional response of microglia against infection via LPS stimulation is one of the most used methods to artificially induce a pathological condition. In Ao *et al* (2021), stimulation of iPSC-microglia-containing forebrain organoids with LPS and opioids led to inflammasome activation and secretion of pro-inflammatory cytokines (IL-1β, IL-18 and TNFα). Furthermore, inflammasome activation was decreased upon LY2828360 treatment. This represented an important novelty, since no studies where microglia phenotypes were rescued by compound treatment have been shown. An increase of IL6 and TNFα release and gene expression was also observed in brain organoids with innately differentiated microglia upon LPS exposure (Ormel *et al* 2018). Infection of brain organoids with ZIKV led to a microglial up-regulation of the viral entry receptor AXL, as well as an up-regulation of the cytokine genes *IL-6*, *IL-1*β and *TNF*α. Interestingly, infection was followed by an increase of synapse elimination by microglia in organoids (Xu *et al* 2021). The reactivity of microglia towards brain lesions has also been assessed in 3D. In Muffat *et al* (2016), researchers co-cultured iPSC-derived microglia with neurons and macroglia to form spheroids. Upon lesion induction via laser in spheroids, microglia morphology changed into an amoeboid shape and cells migrated towards the damaged areas, showing response to cellular damage.

Apart from infection, neuroinflammation has been assessed in organoid models of neurodegenerative disorders. In Abud *et al* (2017), it is shown that iPSC-microglia phagocytose fibrillary beta-amyloid (Aβ) in 2D. Upon transplantation into the brain of an Alzheimer's disease (AD) mouse model, a similar microglial engulfment of Aβ compared to foetal microglia was observed. In AD-cerebral organoid models carrying an amyloid precursor protein gene duplication, the Aβ clearance efficiency from microglia carrying an APOE4 variant was reduced (Lin *et al* 2018). Concerning PD, no studies have been published assessing microglia-mediated neuroinflammation in dopaminergic-rich or midbrain-specific organoids. This could be due to the medium composition incompatibilities discussed in the previous section. Recently, a successful microglia integration method into midbrain organoids has been published (Sabate-Soler *et al* 2022). The same organoid protocol has been used to model neuronal phenotypes in genetic PD (Smits *et al* 2019,

Jarazo *et al* 2021). Using a midbrain organoid model with microglia to study inflammatory phenotypes in a PD context would be of high value.

5. Final remarks

The field of human iPSC-organoids and 3D systems is advancing rapidly. By co-culturing organoids, containing cells with a neuroectodermal origin, together with microglia, which have a mesodermal origin, new and more complex systems are raising. Microglia play a crucial role in the healthy and diseased brain; they maintain brain homeostasis and mediate inflammatory processes. Therefore, the addition of microglia into organoids represents a big step forward into accurately modelling human brain (patho-)physiological processes. The development of different protocols to derive microglia from iPSCs and co-culturing them with iPSC-organoids makes studies more relevant from a personalized medicine perspective.

Interestingly, many studies in 3D culture models have shown results not yet seen in 2D, in particular describing improvements in neuronal electrophysiological properties in the presence of microglia (Fagerlund *et al* 2021, Sabate-Soler *et al* 2022). This suggests that not only 3D models offer an advantage in terms of cell diversity and spatial complexity, but also their high complexity may translate into new discoveries not observed in 2D. Future studies focusing on microglia in 3D systems will bring more information about the advantages of these systems in comparison with 2D, maybe including new observations and discoveries not seen before in other cell culture systems.

Microglia in brain organoids are functional, and respond to inflammatory stimuli such as LPS treatment. Considering that often animal systems do not accurately recapitulate human brain physiological and pathological processes, the functionality and responsiveness of microglia in human organoids represents a big advantage for the scientific community. However, advances should focus on decreasing the manipulation-related stress, which seems to turn microglia in culture into a constantly reactive mode. Protocols where microglia are in the resting mode at normal culture conditions, comparable to the actual situation in the human brain would be of great value to even better model the human brain.

Concerning brain disorder, while there are studies showing AD modelling in organoids with microglia, no studies assessing PD have been published. Drug testing studies in organoids with microglia are scarce. Future efforts should focus on widening this window and assess whether organoids with microglia could be used for compound screening, focusing on inflammatory processes.

The fact that microglia are incorporated into brain organoids is extremely positive, however other mesoderm-derived cell types are still absent in those systems. The development of brain organoids with microglia and a vasculature system would be of great value to study neurovascular interactions *in vitro*.

Overall, the field of organoids and assembloids seems to advance into a direction where neurological disorders could be accurately recapitulated in a personalized medicine manner, which would be of immense value to better understand neuroinflammation and develop treatments.

Data availability statement

No new data were created or analysed in this study.

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