Review

Modelling the Gut-Bain Axis in Neurodegeneration: A Comprehensive Review of Organoids and Organ-on-Chip Systems

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Modelling the Gut-Bain Axis in Neurodegeneration: A Comprehensive Review of Organoids and Organ-on-Chip Systems

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Abstract

According to the World Health Organisation (WHO), conditions linked to the brain account for 28% of the social burden of all diseases, the largest sector, surpassing cancer and cardiovascular disease (CVD). Our incomplete understanding of human neurodegeneration biology is at the center of the devastating impacts it brings on our societies. A fundamental translational effect in those therapies is evident in that none have succeeded in registration-sized clinical trials. The outcome are coexisting therapies that remain largely palliative, managing symptoms or slowing decline but not providing hope for a reversal or cure. Increasing evidence has positioned the gut-brain axis (GBA) as a key modulator of neurodegeneration hallmarks, often inducing or progressing disorders such as Alzheimer's, Parkinson's and Multiple Sclerosis. Traditional research tools fail to recapitulate the accurate physiology of organ systems in humans, leading to the development of organoid technologies and organ-on-a-chip platforms. This literature review comprehensively analyses efforts to model neurodegenerative disorders through in vitro models, evaluating advancements in intestinal, cerebral, GBA, blood-brain barrier and other multi-organ systems. Further, the paper ties back to the known pathophysiology of such diseases and the GBA's influence to evaluate limitations of current disease modelling approaches, offering future directions that enable applications in drug discovery. These technologies mark a transformative shift in methods to understand both the mechanistic causation and therapeutic strategies for previously incurable diseases, expanding the possibilities to improve the lives of millions of diagnosed patients.

Keywords: gut-brain axis; neurodegeneration; organoids; microphysiological systems; organ-on-a-chip; human-specific models; pathophysiology; stem cells; drug screening; alzheimer's

Mechanistic Architecture of the Gut-Brain Axis

The gut-brain axis (GBA) represents the bidirectional pathway between the central nervous system (CNS) and the gut. While the brain controls the secretion of digestive fluids and internal peristalsis, the gut, based on its microbial composition, provides feedback to influence the functioning of the brain. *(Trapecar et al., 2021)*

The gut microbiota encompasses tens of trillions of microbes present in the gastrointestinal (GI) tract, whose presence and abundance dynamically vary. At homeostatic conditions, the gut microbiome maintains the integrity of the mucous membrane through its interactions with the intestinal mucosa *(Groschwitz & Hogan, 2009)*, contributes to the development of endocrine and immune systems *(Farzi et al., 2018)*, and produces neuroactive compounds and neurotransmitters *(Holzer & Farzi, 2014)*. The gut microbiome's formation is a product of various factors, including lifestyle habits like diet and smoking, the external environment, and past medical treatments such as surgery *(Biedermann et al., 2013) (Tyakht et al., 2013) (Jiang et al., 2015) (Rodríguez et al., 2015)*. Therefore, despite colonising the host's intestines from birth, the microbiome experiences constant alterations that have been demonstrated to be associated with overall body health. Specifically, interactions between the gut microbiome and the host have a major impact on the development and further

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progression of diseases, not limited to just disorders related to the gut and the intestines. *(Zheng et al., 2024)*

Dysbiosis-Driven Pathophysiology

Gut dysbiosis refers to alterations in the quality and quantity of microorganisms present in the gastrointestinal (GI) tract. *(Ceppa et al., 2020)* The gut microbiome produces different metabolites that move through direct and indirect passage, both of whom could trigger inflammation and neurodegeneration. *(Logsdon et al., 2017)* With direct passage, specific immune responses, mediated by nerve cells and cellular barriers such as the blood-brain barrier (BBB), protect the brain against microbial invasion. These barriers enable the delivery of nutrients while removing metabolites, ensuring the brain is protected from rapid changes in the biochemistry of the blood. *(Dando et al., 2014)* However, variations in the gut microbiota could impact CNS homeostasis, by inducing cytotoxicity or apoptosis through the release of signalling proteins (peptides). *(Kim, 2008)* Different pathogenic microorganisms target different regions, which are a result of factors in the environment supporting their replication and the distribution of receptors. *(Kristensson, 2011)* Therefore, those linked to the brain region pose a risk to brain health and could potentially cause disease. Indirect passage, on the other hand, occurs in the case of soluble biochemicals produced by the microbiome, which reaches the BBB, and through it, the brain. *(Matsumoto et al., 2013)* This also alters homeostasis, and can show neurotoxic effects.

At a larger level, immune mediation causes an increase in intestinal permeability, resulting in the movement of microorganisms and other components (for instance, lipopolysaccharides) from the gut to the circulatory system. *(A. Kohler et al., 2016)* Lipopolysaccharides are a component of the external cell membrane of Gram-negative bacteria, and are often found in neurodegenerative conditions. *(Asti & Gioglio, 2014) (Tufekci et al., 2011)* Pathological conditions caused by such components are a result of the triggering of systemic inflammation across the body in their presence, with the release of pro-inflammatory cytokines increasing BBB permeability, thereby causing brain damage (either directly or indirectly). *(de la Fuente-Nunez et al., 2017)*

Another example of this is BMAA. Certain species of Cyanobacteria, present in the GI tract, produce Beta-methylamino-L-alanine (BMAA), a non-protein neurotoxic amino acid suspected of playing a role in neurodegeneration. *(Burton, 2013) (Karlsson et al., 2014)* This has been backed by the identification of major concentrations of BMAA in the brains of Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) patients. BMAA is an excitotoxin known for its activation of metabotropic glutamate receptor 5, resulting in an increase in oxidative stress. *(Ballatori et al., 2009)* This results in a disruption of the otherwise controlled production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) by neurons and glial cells present in the brain. *(Brenner, 2013)* One research group used a new antibody to study BMAA accumulation in the brain, discovering that toxicity caused by BMAA results in a neurotoxic action on motor neurons. This indirectly leads to the activation of glial cell dysfunction, which, in turn, furthers neurotoxicity. *(Tan et al., 2017)*

Our previous understanding of the role of the gut microbiome on neurodegenerative diseases was validated by a clinical study, which highlighted, in support of existing research, the altered state of the gut microbiome in cases of AD. Alterations include changes in the level of abundance of bacterial taxa such as *Firmicutes*, *Actinobacteria* and *Bacteroidetes*. One key sector of this correlation is between the abundance of microbes and pathological biomarkers in cerebrospinal fluid (CSF). For instance, a decrease in *Bifidobacterium* could be a cause for the reduction seen in amyloid-Beta in the CSF. Since the CSF surrounds the brain and spinal cord, the amount of amyloid-Beta present in it assists our estimation of how much is being cleared from the brain. *(Vogt et al., 2017)* Therefore, higher levels in the CSF suggest lower accumulation in the brain, demonstrating the protective role played by *BIfidobacterium* in maintaining gut homeostasis, preserving intestinal permeability and managing immune responses. *(Ruiz et al., 2017)*

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Integrated Signalling Pathways of the GBA

The gut-brain axis is the result of a complex network system, integrating neural, endocrine and immune pathways. The bidirectional connection seen between the two exist via three key connections: the hypothalamic-pituitary-adrenal axis, the central nervous system and the enteric nervous system (ENS). *(Mayer et al., 2022)*

Neural Circuits Mediating Gut-Brain Signalling

Neural pathways contributing to the gut-brain interplay include the CNS, which regulates activity of gastrointestinal cells through the autonomic nervous system (ANS). *(Wang et al., 2023)* The ANS is a component of the peripheral nervous system, and is divided into sympathetic and parasympathetic. Of its various functions, the ANS plays a critical role in maintaining the permeability of the intestinal cells, producing gut antimicrobial peptides and modulating the mucosal immune response against pathogens. *(Martin et al., 2018) (Mayer, 2011)* The ANS integrates with the gut and the brain through the transmitting of efferent and afferent signals. Efferent signals travel from the CNS to the intestinal walls, while afferent signals are transported from the opening of the intestinal tract to the CNS through enteric, spinal and vagal pathways. *(Carabotti et al., 2015)* The ANS is also capable of indirectly receiving signals from the gut microbiota through the release of metabolites and bacterial compounds. *(Bonaz et al., 2018)*

Amongst the various other pathological conditions, stress is especially harmful to the ANS in relation to the GBA, since it activates a negative feedback loop that eventually results in dysbiosis. Stress stimulates the sympathetic system, while inhibiting the vagus nerve. *(Wood & Woods, 2007) (Taché & Bonaz, 2007)* This leads to an increase in the secretion of pro-inflammatory cytokines, promoting local and systemic inflammation. *(Marsland et al., 2017)* Inflammation further results in the stimulation of the vagus nerve activates anti-inflammatory pathways, completing a feedback mechanism that ensures inflammation caused by the cytokines are reduced. However, this combination of sympathetic activation and parasympathetic (vagal) inhibition disrupts homeostasis in the gut, resulting in dysbiosis and an increase in gut permeability. *(Bonaz et al., 2013)*

The enteric nervous system acts as a 'second brain', consisting of a massive network of nerve cells scattered across the intestinal walls, and working in the production of neurotransmitters. *(Wang et al., 2023)* While acting as the main division of the ANS, the ENS works towards different functions, including modulating motor activities and secretion in the gastrointestinal system, considering 90 to 95% of the body's serotonin is produced by enteroendocrine cells present in the tract. *(Kim, 2008)* The ENS also supports the maintenance of homeostasis of the GI tract alongside the brain, microbiota, endocrine and immune system.

Vagal routes are extremely significant, emerging from the brain up until the intestinal wall via the intestines, and connecting to the ENS through synapses. *(Amirifar et al., 2022)* Disruptions of vagus nerves linked with the gut impact various brain conditions, including neurological development, stress and anxiety. In fact, this demonstration of the gut's communication with the brain has been shown to influence cognitive change, to the extent of impacting behavior. *(Keute & Gharabaghi, 2021)* Gut microbiota and their metabolites often impact neurodevelopment and cognition through neurons of the ENS, which then connect with vagus nerves corresponding to various functions, including even sleep. *(Sgritta et al., 2019) (Bonaz et al., 2018)*

To fully understand the extent of this correlation, we can understand the interplay between the gut and the brain by taking the case of obesity. Changes in diets, including high-fat and high-carbohydrate diets disrupt the diversity of the gut microbiota. *(Aron-Wisnewsky et al., 2021)* In turn, this loss of stability impacts the hypothalamic function, with phenomena such as inflammation and gliosis being triggered through vagal signalling. This stimulation leads to the onset of obesity, a lifestyle disease whose prevalence is rapidly rising in recent years. *(Abdel-Haq et al., 2019)* This example demonstrates how chronic ailments, like obesity, believed to be solely restricted to the

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workings of the digestive systems, are in fact formed and regulated through mechanisms involving the nervous system.

Neuroendocrine Pathways and Stress Signalling

The second of the three connections, namely the hypothalamic-pituitary-adrenal (HPA) axis, represents the foundation for the neuroendocrine pathway, using hormones and neuropeptides to regulate various activities, both across the body and specific to the GBA. *(Frankiensztajn et al., 2020)* These range from digestion and immunity to sexual and stress responses *(Foster & McVey Neufeld, 2013) (Bercik et al., 2012)*; however, of great significance in the context of the gut-brain axis is its relevance in intestinal motility and inflammation. *(Frankiensztajn et al., 2020)* For the most part, the stress-related alterations in the gut are mediated by Corticotropin-releasing hormone (CRH) receptors, present in both the brain and the gut. However, under stress, neurons and neuroendocrine signaling molecules, stimulated by the tension, can result in gastrointestinal dysfunction, through changes in the gut microbiome's composition. *(Vodička et al., 2018) (Zhou & Fang, 2018)* Further, such inflammatory responses can activate the HPA axis, thereby impacting the brain. An increase in Gram-negative bacterial growth *(Lyte et al., 2010)* results in a rise in the permeability of the intestinal barrier, resulting in a translocation of bacterial taxa across the inner space of the intestines. *(de Punder & Pruimboom, 2015)*

A prime example of the gut microbiota's role in the brain through the endocrine pathway is short-chain fatty acids (SCFAs), produced by bacteria in the gut. These SCFAs induce the release of GI hormones, including glucagon-like peptide 1, whose signals reach the brain through circulation across the body. These signals have demonstrated influences in memory, emotions and cognitive function, among others. *(Dalile et al., 2019)*

Immune-Mediated Modulation of the GBA

The immune system has a significant influence on the gut and the brain, both as individual elements and as an interconnected axis. The gastrointestinal tract contains the highest number of immune cells in the body. The mucosal immune system, representing the inner lining of organs such as the stomach, provides direct immunity to intestinal sites, establishing a barrier that prevents the invasion of harmful microbiota. *(D'Amelio & Sassi, 2017) (Sittipo et al., 2018)* Overall, the immune system is critical in supporting the balance of gut microbial populations, by eliminating harmful microbe populations and aiding the proliferation of beneficial ones. From the opposite lens, an unbalanced gut microbiome also has the potential of forcing a change in the expression of inflammatory genes. *(Minter et al., 2016)*

In vivo models of neurodegeneration have demonstrated the effects of the gut microbiota on the functioning of immune cells in the CNS. Specifically looking at AD as an example, we see a rise in inflammation-related proteins, with alterations in the levels of anti-inflammatory and pro-inflammatory cytokines in circulation. *(Rothhammer et al., 2018)* Microbiota in the intestine can induce inflammation, as demonstrated earlier, alongside the aggregation of cerebral amyloid-Beta, clearly tying back to this point. *(Pistollato et al., 2016)*

The GBA plays a critical role in the regulation of inflammation, primarily through the systemic-humoral pathway, which supports the delivery of immune factors. Ailments such as infections and gut dysbiosis can lead to the release of gut-derived inflammatory factors, including tumor necrosis factor-alpha (TNF-alpha) and interleukin-6 (IL-6). *(Parker et al., 2019)* This causes greater intestinal permeability, disrupting the blood-brain barrier (BBB). This disruption activates local immune cells of the BBB, triggering neuroinflammation, demonstrating the gut's role in immune responses in the brain. Gut immune cells directly influence brain responses to GBA inflammation through the cellular immune pathway. Inflammatory immune cells, once activated, can move to the CNS, often worsening neuroinflammation in brain disorders such as Parkinson's and Multiple Sclerosis (MS). However, the role of immune pathways also exists in a flipped version - alterations in HPA axis activity due to inflammation result in the release of glucagon and corticosterone, impacting gut function. *(Cryan et*

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al., 2019) In the case of Alzheimer's Disease (AD), BBB integrity is compromised as a result of inflammatory cytokine release and bacterial translocation. *(Qu et al., 2023) (Grabrucker et al., 2023)*

SCFAs were previously described while discussing endocrine pathways. They also play a key role in neuroimmune regulation as part of the gastrointestinal tract. A prime example is butyrate, a SCFA that supports the regulation of the gut immune system *(Furusawa et al., 2013)*, alongside the expression of antimicrobial peptides *(Guaní-Guerra et al., 2010)*. These provide evident cases where biological components produced by the gut microbiome directly play a role in the immunity of the gut, and through it, its connection to the brain via the given pathways.

Role of the Blood-Brain Barrier in Gut-Brain Communication

The blood brain barrier (BBB) is a membrane that protects the CNS from harmful substances, namely pathogens and toxins, present in the blood. *(Wu et al., 2023)* Acting as a regulatory boundary between the brain and blood, it supports the maintenance of homeostasis, and aids in key processes of communication and nutrition. *(Logsdon et al., 2017)* The properties of the BBB are largely a result of the endothelial cells (EC) present, enabling interactions between mural, immune, glial and neural cells, which thereby interact with the neurovascular unit. *(Muoio et al., 2014) (McConnell et al., 2016)* The BBB is primarily composed of neurons, glial cells (including microglia, astrocytes and oligodendrocytes), brain endothelial cells, smooth muscle cells, and pericytes rooted in the extracellular matrix (ECM) of the brain. *(Zlokovic, 2011)* A phospholipid bilayer of plasma membrane selectively permits the movement of small gaseous molecules, lipophilic agents and small polar molecules. While most hydrophilic molecules are supported by transporters that enable their transfer through blood, those larger in size must move through a cellular process termed endocytosis (internalising of molecules or other cells). Tight junctions between endothelial cells regulate pathways for the movement of molecules and ions between cells. *(Stamatovic et al., 2008)*

GBA Contributions to Neurodegenerative Disease Mechanisms

The composition of the gut microbiome can change throughout an individual's life, reflecting a dynamic nature that is critical to understanding dysbiosis, which is further tied to pathological conditions. *(Thursby & Juge, 2017)* An imbalance in gut bacteria results in the activation of the immune system, which increases the permeability of the gut barrier and inflammatory mediator levels, thereby promoting neurodegeneration and neuroinflammation. *(Kowalski & Mulak, 2019) (Groschwitz & Hogan, 2009)* This has led to rising scientific investigation towards the links between dysbiosis to pathological states, including anxiety and depression *(Wang & Kasper, 2014) (Foster et al., 2017)*, autism spectrum disorders *(Yarandi et al., 2016) (Li & Zhou, 2016)*, schizophrenia *(Nemani et al., 2015)*, and neurodegenerative disorders *(Roy Sarkar & Banerjee, 2019)* such as Alzheimer's *(Vogt et al., 2017)*, Parkinson's *(Sampson et al., 2016)* and Multiple Sclerosis *(Cekanaviciute et al., 2017)*. Even more fascinating is the discovery of novel drugs for neurodegenerative disorders (such as AD), with the ability of reversing cognitive decline by suppressing gut dysbiosis *(Wang, Wu, et al., 2018)*, reinforcing the significance of such an axis.

Pathophysiology of Neurodegenerative Disorders

Before working through the role of organoids in modelling how communication pathways (the GBA) influence neurodegenerative disorders, it becomes critical to first understand the pathological basis of their presence.

Alzheimer's Disease involves a progressive decline in memory alongside cognitive deterioration. The most damaging element, however, is the irreversible nature of this degeneration. The physiology of Alzheimer's is primarily characterised by the destruction of nerve cells, along with neural connections between them. This results in a loss in brain mass, particularly in the cerebral cortex which is primarily affected by the diseases. Key hallmarks of the disorder include extracellular plaques contributing to a loss in mental faculties (such as the amyloid-Beta plaques previously

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described) *(Shankar et al., 2008)* and intracellular tanglement of neural fibres *(Reitz et al., 2011)*. Many of these characteristic features are the result of protein misfolding, further listed as a triggering step for synaptic dysfunction, neuronal degeneration and neuroinflammation. *(Bi et al., 2021)* AD can further be classified into a familial (FAD) or sporadic (SAD) nature. Here, FAD is predominantly caused by gene mutations encoding amyloid precursor protein (APP) and presenilin 1 (PSEN1). *(Choi et al., 2016)* SAD, a more common late-onset, polygenic form of Alzheimer's, arises in individuals without mutations, instead driven by complex interactions between aging, environmental and metabolic factors. *(Tanzi, 2012) (Van Cauwenberghe et al., 2015)* Calls for increased financial resources, goods and services, and long-term care for AD patients from the International Alzheimer's Disease Association report accentuate the need for developments in disease understanding and therapeutic approaches.

Parkinson's Disease involves the impairment of motor control, caused by the presence of dopaminergic neurons in the substantia nigra. A primary hallmark for the disease is the accumulation of a-synuclein (a-syn) *(Dauer & Przedborski, 2003)*, leading to an increase in intestinal permeability and the potential movement of bacteria and microbial inflammatory compounds into the bloodstream *(Perez-Pardo et al., 2017)*. While still a hypothesis, some research studies have described the role of a-syn in gastrointestinal dysfunctions, namely the damage caused by its accumulation to enteric neurons present in the enteric nervous system. *(Gelpi et al., 2014) (Gold et al., 2013) (Forsyth et al., 2011)*

Multiple Sclerosis is an autoimmune disease involving progressive neuronal loss, through the atrophy, or gradual decline, of various elements present in nerve cells (such as myelin sheaths, axons and nerve fibres). Certain studies have brought to focus certain bacterial taxa whose presence varied in Multiple Sclerosis patients from patients without the disorder. Species such as *Akkermansia muciniphila* and *Acinetobacter calcoaceticus*, known for bringing about pro-inflammatory responses, were higher in MS patients, while *Parabacteroides distasonis*, a microbe known for stimulating anti-inflammatory interleukins, was reduced. *(Cekanaviciute et al., 2017) (Mowry & Glenn, 2018)* This highlights the strong possibility of an association between these microbes present in the gut and the disorder.

Disease-Specific Organoid Systems

Organoids are 3-dimensional (3D) models of organs, made of cells that can self-organise. These organoids are extremely useful in helping us understand human anatomy and physiology and pathogenesis, along with more effective treatments for diseases through drug testing personalised medicine technologies. *(Zheng et al., 2024)*

2D cell cultures have served as a method for studying cell biology for multiple decades. However, fundamental differences cause a separation in the effectiveness of 3D cell environments from 2D cultures. Firstly, the increased expression of cell-associated markers empowers an improved spatial understanding of the location of such molecules in 3D cultures. Taking the brain as an example, the improved localisation of nerve cell markers in 3D environments supports a greater representation of synaptic connections. *(Bi et al., 2021)* Secondly, 3D cultures are more faithful in the ability to mimic organ environments, since 2D cells (even in co-cultures) only spread horizontally. *(Cullen et al., 2011)* Finally, a 3D environment supports connections between cells, enabling an enhanced simulation of their interactions as they would be in vivo. For instance, 2D monolayers prevent synaptic connections from forming due to large spaces between neural cells. On the contrary, 3D cell environments hold nerves together as bulbous masses, supporting intercellular contacts in all directions. *(Seidel et al., 2012)* The presence of tight connections between cells in in vitro cultures is critical to enabling accurate simulation of disease hallmarks. For instance, the inability for AD 2D-culture cells to produce amyloid-Beta accumulations rendered it difficult to trigger further pathogenic reactions, limiting the model's ability to replicate the disease. *(D'Avanzo et al., 2015)* In these ways, we see how the use of organoids are advantageous in our pursuit of a greater understanding of cell and organ morphology, function and pathology.

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Organoids are often constructed with adult stem cells, ensuring the genetic and epigenetic mutations of the host are preserved. *(Schreurs et al., 2019)* However, recent research and scientific breakthroughs have also enabled the use of stem cells that are either sourced from embryos (ESCs) or differentiated from pluripotent states (PSCs). *(McCracken et al., 2014)* These remain in development, being more complex and time-intensive; especially in the case of PSCs, challenges with genomic instability due to exposure of reprogramming factors makes it challenging to assess the accuracy of the resulting organ model. *(Günther et al., 2022)*

Intestinal Organoids

Intestinal organoids are 3D models of the human intestines created from stem cells, composed of a range of intestinal epithelial cell types. These include Lgr5+ intestinal stem cells (primary self-renewing cell population for epithelial lineages); hormone-producing enteroendocrine cells (specialised hormone-producing cells); mucus-secreting goblet cells (secrete mucins to maintain barrier integrity); absorptive enterocytes (responsible for nutrient absorption); and paneth cells (secrete antimicrobial peptides as protection from pathogens). *(Takahashi et al., 2021)* Most simple intestinal organoids are limited in their application and accuracy due to the absence of gut microbiota. Some groups aimed to integrate certain microbes for the simulation of disease or the study of their role. For instance, one group created an air-liquid interface (ALI) culture system, which replicated the entire life cycle of Cryptosporidium parvum, a bacterial species that causes Cryptosporidiosis, a parasitic disease, in the GI tract. *(Wilke et al., 2019)* Another set of researchers developed a co-culture model of human intestinal organoids with genotoxic pks + E. coli, with findings indicating a direct association between exposure to the latter and oncogenic mutations, such as colorectal cancer. *(Pleguezuelos-Manzano et al., 2020)* Finally, outside the realm of pathology, co-cultures between intestinal organoids and microbe-delivered metabolites have indicated the role of these metabolites in modulating endoplasmic reticulum (ER) stress. *(Ke et al., 2021)* This not only highlights the diversity of intestinal organoid applications, but also the importance of integrating the gut microbiome in such models for a wider scope of applicability and higher degree of replicability.

One key challenge with such culture studies are their short lifespan, lasting only hours or days. These prove to be inadequate for simulating chronic conditions linked to the gut microbiome. *(Puschhof et al., 2021)* One group was able to devise a framework for a co-culture by injecting a single bacterial species over a 5-month period. *(Pleguezuelos-Manzano et al., 2020)* Further, gut-on-a-chip systems, discussed in a later section of this review, have shown promise in rendering complex bacterial communities or single species into these in vitro models for durations upto weeks. *(Jalili-Firoozinezhad et al., 2019)* Long term co-cultures are critical for enabling proper study, necessary for a deeper understanding of both disease progression and drug efficacy.

Immune Cell Co-Culture Systems

In order to work towards a more accurate model of the human intestines, their interactions with other body systems and parts is crucial. One such example, as was previously mentioned, is the immune system. Various groups have experimented with different methods to create co-culture systems between intestinal organoids and immune cells. For instance, a novel technique to nurture tumor-reactive T cell formation involved a co-culture of peripheral blood lymphocytes and tumor organoids from the same patient. These patients had repair-deficient colorectal cancer and non-small cell lung cancer. The given co-culture enabled the proliferation of tumor-specific T cell growth. *(Dijkstra et al., 2018)* Another co-culture, formed between intraepithelial lymphocytes (IELs) and intestinal epithelial cells (IECs), examined the interaction between immune cells and the gut epithelium, by specifically analysing these two cell types. *(Neal et al., 2018)*

The gut immune system is vast, composed of structures that are referred to as gut-associated lymphoid tissue (GALT). The immune system in the gut is distributed across the epithelial layer (innermost lining of the intestine), lamina propria (connective tissue layer housing immune cells and blood vessels), and lymphoid follicles (clusters of gut-associated lymphoid tissue -GALT-). In 2023, a

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paper published findings from a study involving the development of the first intestinal organoids with a functional immune system, modelling GALT-like structures. The organoid was able to mimic immune responses post-bacterial infection, including an activation of immune cells, the production of immunoglobulin A (IgA) and an increase in M cells. *(Bouffi et al., 2023)*

Modelling the Enteric Nervous System

Over the years, research groups have used various techniques and methodologies to develop a functional enteric nervous system in intestinal organoids. One group created a co-culture between neural crest cells and human intestinal organoids, integrated the ENS and enabled a study of disorders linked to it. *(Workman et al., 2017)* Other researchers have used human embryonic stem cells to create colonic organoids, ensuring both enteric nerves and blood vessels are incorporated. *(Park et al., 2020)* A unique approach adopted in one study created intestinal organoids from three germ layers, namely epithelial precursors, mesenchymal and enteric neuroglial. The incorporation of all three layers produced differentiated glands in the organoids, enclosed in functional enteric neurons. *(Eicher et al., 2022)*

Engineered Vascularised Organoid Systems

Vascular structures present those biological components linked to vessels, with the primary responsibility of enabling the movement of blood. Such elements are responsible for key functions in the body, including waste removal, delivery of nutrients, and the overall accuracy in mimicking human body conditions. *(Rafii et al., 2016)* One research group used single-cell RNA sequencing to expose the vascular endothelium within human pluripotent stem cell (PSC)-derived organoids. This modification of culture conditions enabled the differentiation of endothelial cells, supporting the recreation of vascular structures in lab conditions. *(Holloway et al., 2020)* The example of colonic organoids listed under the ENS, alongside enteric nerves, also contained blood vessels, and therefore, vascularisation. *(Park et al., 2020)*

One study focused on outlining a general procedure for the formation of vascularised organ buds across tissue types. The method involved mesenchymal cell-driven condensation, where pluripotent stem cell-derived tissue samples were combined with endothelial and mesenchymal stem cells (MSCs). This set-up took advantage of the unique tendency for MSCs to guide the formation of condensed structures as result of surrounding stiffness. *(Takebe et al., 2015)*

Outside the sphere of traditional 3D organoids, novel technologies such as microfluidics have also been leveraged to support the vascularisation of organoids. One such example is the IFlowPlate, a microfluidic platform that can produce as many as 128 vascularised colon organoids. These organoids are under the constant supply of blood through fluid passages, as compared to static conditions, while also not requiring external pump systems. The platform was even used in a colon inflammation model, demonstrating an innate immune function as seen in in vivo conditions. *(Rajasekar et al., 2020)*

Advances in Bioengineering and Co-Culture Technologies

This section has broadly reviewed the techniques and use cases of intestinal organoids; however, there remain certain examples outside this scope which are significant in their own right.

Apart from the co-cultures mentioned above, there is another that has been experimented with: between intestinal organoids and mesenchymal cells. One study looked at how mesenchymal niche-derived neuregulin-1 (NRG1) led to intestinal stem cell proliferation and the regeneration of damaged epithelium layers. This led to an understanding that intestinal organoids could enable the study of tissue repair and regeneration through stem cells. *(Jardé et al., 2020)* Another group looked at mouse genetics and high-throughput sequencing using murine organoid models, identifying TGF-Beta1 as a key driver of the shift of the intestinal epithelium to a prenatal-like regenerative state. The

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protein was therefore highlighted as having the potential to act as a pretreatment for patients with damaged colon tissues. *(Chen et al., 2023)*

Another growing field across the medical sciences has been the advent of 3D bioprinting, which involves the ability to craft and assemble biological and non-biological materials in structures defined by computer-aided tools. *(Chen et al., 2023)* Some research groups have applied this concept to the growth of organoids, including intestinal ones. For instance, one study employed stem cell components traditionally used to form organoids as the material for centimeter-scale tissues. The end structure formed included elements such as lumens, tubular intestinal epithelia containing crypts and villus domains, and vasculature, all of whom were self-organising. *(Brassard et al., 2020)* In fact, bioprinting-assisted tissue emergence (BATE) is a specific technology working at the convergence of organoids and biofabrication to enable and manipulate tissue self-organisation. *(Moldovan et al., 2017)*

Cerebral Organoids

The brain is the central organ of the nervous system, protected by various structures and systems, including the skull and the blood-brain barrier. When inflammation changes the morphology of these protective barriers, pathogens can reach the brain. The brain is made primarily of two cell populations, namely the nerve cells and glial cells. *(Raimondi et al., 2020)* Nerve cells, or neurons, are made of a cell body at the center, dendrites that pick up messages from other nerves and the axon transmitting these impulses to the end of the cell. Neurons communicate through synapses, which are connections that support the passage of these nerve impulses. There are two types of signals that can be transmitted: chemical triggers for signals created from the release of neurotransmitters, and electrical signals formed by the movement of ions across the neuronal membrane, creating action potentials. *(Kandel, 2013)* Glial cells, the second type of cell population in the brain, support neurons by providing protection, structural support and nutrition. Glial cells exist in various types: astrocytes, responsible for maintaining homeostasis and promoting neuronal survival; microglia, which protects the brain from infections through immune system-like activity; oligodendrocytes, critical for the formation of myelin sheath over neurons in a process termed neuronal myelination; and ependymal cells, which help produce cerebrospinal fluid. *(Sven Jäckel et al., 2017)*

3D Cerebral Organoids

For a long time, human brain studies were limited to postmortem or biopsy investigations, rendering in vivo observations of CNS pathology unfeasible. However, progression in in-vitro studies in recent decades have enabled an improved understanding of neurological disorders. Considering the complexity of the brain's morphology as described above, modelling the entire brain represents a massive challenge, given the surface dimensions and sheer cell population diversity. Initial versions of the brain models worked on single cell cultures, failing to consider the interactions between nerve and glial cells. However, recent studies have shifted their focus to cell-cell interactions and the accurate mimicry of human tissue. *(Raimondi et al., 2020)* Further, 3D cultures have improved the ability to clinically translate such brain models. *(Hopkins et al., 2015) (Hasan & Berdichevsky, 2016)*

Reprogramming technologies through the expression of four transcription factors aid the differentiation of hiPSCs into mature somatic cells, enabling their development into 3D neuroepithelial tissues. *(Takahashi et al., 2007)* Such neuroepithelial tissue, displaying intact architecture and containing genetic elements of the human brain, still lacks structure and coordination between brain subregions. *(Brennand & Gage, 2011)* New models were developed to self-organise and self-develop, ensuring preservation of subregional architectures. *(Eiraku et al., 2011)* These advances were supported by the production of a gel-like extracellular matrix, Matrigel, which crafted an appropriate environment for organoid self-organisation. *(Sato et al., 2009)* By 2013, hiPSCs were differentiated into a whole cerebral organoid. *(Ma et al., 2013)* The differentiation of these stem cells resulted in a neuroectoderm structure, simulating the cerebral cortex's early development stage. *(Lancaster & Knoblich, 2014)* RNA sequencing technology employed on such 30-day-old human

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cerebral organoids demonstrated the presence of similar gene expression patterns as fetal brains during pregnancy, highlighting the ability of such in vitro tissue culture studies in replicating brain morphology. *(Dang et al., 2016)* Oxygen and nutrient distribution to generate forebrain, midbrain and hypothalamic organoids was also made possible using bioreactor technology. *(Qian et al., 2016)*

One study aimed to model the brain cortical architecture, reproducing the compartmentalisation of gray and white matter through protein-based porous layers combined with collagen gel. This supported the formation of 3D axon connections, accurately representing in vivo brain structures. *(Tang-Schomer et al., 2014)* Another group recommended a layer-based approach using bioprinted gellan gum; however, the selection of printable materials and challenges with structural collapse have made the advent of bioprinting brain organoids unlikely. *(Lozano et al., 2015)* One group cultured human neural progenitor cells to over-express key AD genes (APP or APP and PSEN1). *(Choi et al., 2014)* Results from the study demonstrated higher levels of toxic amyloid-Beta deposits and p-tau in 3D, as compared to 2D, cultures. Such research supports the conclusion that in vitro 2D environments are more physiologically relevant, and therefore valid, for further studies and AD drugs testing. *(Zhang et al., 2014)*

AD Cerebral Organoids

Various methods have been used to create AD cerebral organoids: the use of Atfin-5, an A-Beta-42 agonist, to induce A-Beta-42 peptide production (resembling the brain of a SAD patient) *(Pavoni et al., 2018)*; using iPSCs derived from FAD patients to produce the organoids *(Gonzalez et al., 2018)* *(Raja et al., 2016)*; and the conversion of APOE3 to APOE4 to create differentiated cerebral organoids from SAD patient-derived iPSCs. *(Lin et al., 2018)*

To model FAD, one group used pluripotent stem cells from diseased patients that contained either APP duplications or PSEN1 mutations. These were differentiated into cerebral organoids, with the resulting models accurately depicting the accurate timeline of amyloid-Beta aggregates and subsequent P-tau level rises seen in human FAD processes. *(Raja et al., 2016)* Another study employed stem cells carrying a missense mutation, A246E, in the PSEN1 gene, producing organoids that exhibited a degree of NFTs and cellular apoptosis proportional to protein aggregates accumulation. *(Gonzalez et al., 2018)* The extracellular matrix plays a pivotal role in the human CNS, enabling their generation, migration, differentiation and synaptic plasticity. Nerve cells were modelled, exhibiting an upregulation in syndecan-3 (responsible for mediating cell proliferation and inflammation); the downregulation of metalloproteinase-2 (responsible for neurogenesis and axon regeneration); and downregulation of metalloproteinase-3 (ensuring synaptic remodelling and degradation of the amyloid-Beta protein). Each of these molecules reflect how changes in ECM components are linked to neuronal damage. *(Yan et al., 2018)*

Other studies have worked to model SAD, including the use of Atfin-5 to produce cerebral organoids from human stem cells. Specifically, the introduction of Atfin-5 disrupted the physiological balance between amyloid-Beta-42 and -40, resulting in amyloid-Beta aggregation over time in a manner similar to what was seen in FAD organoids. *(Pavoni et al., 2018)* Another group used the genetic editing tool CRISPR/Cas9 to induce a mutation from the APOE3 to APOE4 protein. The increased levels of amyloid-Beta and P-tau to APOE4 organoids compared to APOE3 organoids demonstrates the ability of such models to replicate SAD hallmarks. *(Lin et al., 2018)*

Animal Models

Three primary types of animal models were seen employed in replicating Alzheimer's Disease: aging models, transgenic models, and models administered with the AD-inducing agents. As an important pathogenic factor of the disease, models split into natural and rapid aging help demonstrate the relationship between the rate of aging and the onset of AD. *(Bi et al., 2021)* Studies have shown the presence of AD pathological characteristics (such as astrocytic responses to oxidative stress) in rapid-aging models. *(Takahashi, 2010)*

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Transgenic models involve the introduction of exogenous human-gene mutations, a result of the accelerated discovery of AD genes (such as APP, PS and ApoE). However, the absence of characteristics like neurofibrillary tangles, tau hyperphosphorylation and neuronal apoptosis highlight key shortcomings of such models. *(Masliah et al., 2001) (Van Dam et al., 2003)* Further, a comparison between murine models and human patients highlight key differences in AD progression. For instance, while brain atrophy occurs at early development of transgenic animal models, neuronal apoptosis primarily occurs with an increase in age in human patients. *(Perrin et al., 2009) (Fan et al., 2008)*

The administration of AD-inducing agents aids the simulation of SAD patients, given 90% of such cases are believed to result from exogenous environmental factors. Mouse SAD models created through the introduction of harmful substances (such as aluminum) have failed to show tau hyperphosphorylation, illustrating a key limitation of such AD animal models. *(Balducci & Forloni, 2025) (Singh et al., 2018)* Other limitations include the differences in brain physiology between the species, leading to a lack of proper mimicry; high mortality; and poor representation of abnormalities across structural, cellular and protein levels.

3D-Printed Brain Models

Researchers have also worked on the notion of 3D-printed brain models, addressing key challenges such as lengthy times to produce a prototype and the lack of standardisation across the entire process of culturing and organoid growth. *(Mahumane et al., 2018) (Rao et al., 2021)* This technology supports the creation of in vitro models using various materials, which include biological components such as living cells. For instance, models of the CNS which include construction of the given structure along the z-axis, represent development perpendicular to a flat 2D culture. *(Cadena et al., 2020) (Liu et al., 2021)* An additional benefit of this application of 3D printing is the ability to customise the designs of organoids, supporting the accurate replication of specific regions or processes in the brain. Finally, a recent space of development has been bionic brain chips, or a brain-computer interface, which can support a detailed study of the physiological and pathological processes taking place in this complex organ.

Vasculature in Cerebral Organoids

Brain vasculature plays a critical role in both accumulating and clearing neurotoxins found in various neurodegenerative diseases, such as AD. The neurovascular unit in particular is critical to the BBB, limiting entry of blood components and neurotoxicants alongside a supply of nutrients and oxygen. Organoids where nutrients and oxygen did not reach its innermost regions faced volume shrinkage and cellular apoptosis after 180 days, making them too young to simulate a real AD brain. *(Kelava & Lancaster, 2016)* Further, when blocked, neural and vascular toxic substances may accumulate in the brain, resulting in pathological changes, signifying its relevance to neurodegenerative disorders. *(van Norden et al., 2012)* For instance, brain blood flow (hemodynamics) is linked to amyloid-Beta production, APP synthesis and tau phosphorylation. *(Zlokovic, 2011)* Therefore, the absence of BBB renders the simulation of diffusion and absorption naturally seen in the brain difficult for cerebral organoids.

Various methods have been employed to integrate the vascular system in cerebral organoids. One approach involves implanting a mature human cerebral organoid in vivo (into a mouse brain), ensuring vascularisation and functional neuronal and synaptic activity through the inclusion of grafts. *(Mansour et al., 2018)* Another group used differentiated hPSCs to differentiate into vascular endothelial cells, enabling the appearance of a vascular system in a few weeks. *(Pham et al., 2018)* Finally, a study used CRISPR/Cas9 gene editing to express the human vascular endothelial gene ETS variant 2 in hESCs, combining them with normal hESCs to produce cerebral organoids with a vascular system. *(Cakir et al., 2019)* Each of these techniques represent unique methods to produce a vascularised cerebral organoid.

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Novel Cerebral Organoid Applications

Cerebral organoids reflecting different neurodegenerative disorders foster the exploration of new drugs. For instance, one group evaluated potential drugs in FAD organoids, with results demonstrating DAPT inhibited amyloid-Beta-42 aggregation, thereby improving neuronal survival. Heparinase III, another molecule, degraded heparan sulfate proteoglycans in the ECM, preventing a-Beta-HSPG interactions, resulting in a decreased tau aggregation. *(Yan et al., 2018)*

Organoids themselves could serve as a treatment for AD. One study used grafts to integrate human cerebral organoids into monkey brains, demonstrating the ability of these organoids to survive in the host cerebral cortex. These demonstrate the potential of organoids, possessing the ability to create BBBs and connections with other organs, as a novel approach for reconstructing neural circuits post-neural death caused by AD. *(Kitahara et al., 2020)* Another group transplanted cerebral organoids into murine brains, demonstrating synaptic connections with host neurons and the acquisition of electrophysiological maturity. *(Dong et al., 2020)*

Biobanking

Biobanks are large collections of biospecimens for primary application in the medical space, often being linked to specific health information of given specimens. *(Annaratone et al., 2021)* The maintenance of organoid biobanks are critical in ensuring standardisation and replicability of models in future experiments and studies. To realise the importance of such conformity, we must first determine the key features of these biobanks, and how they influence the organoid's resulting morphology and functionality.

Firstly, biobanks must include information on the patient and the sample, since organoid cultures have been shown to retain characteristics of their donors. *(van de Wetering et al., 2015) (Zachos et al., 2015)* By providing detailed donor information, from basic characteristics of gender, age and potential diseases, to additional data on family history, diet and medication, we can evaluate the utility of the generated organoids to different cases. This further enables the application of organoids as personalised treatment approaches. Apart from the above donor information, gaining insight on the tissue sample, including the tissue type, time from obtaining the tissue and the in vivo microenvironment, all impact cellular function, and therefore, the organoids' features. *(Perrone & Zilbauer, 2021)*

Culture conditions and growth are critical in influencing the molecular processes taking place in organoids. For instance, Wnt3a, a critical component of human organoid culture mediums originally varied between studies, impacting organoid growth. *(Pleguezuelos-Manzano et al., 2020)* However, through the development of a new surrogate Wnt ligand able to bind to the Wnt receptors, the reproducibility of organoid work using the protein was greatly enhanced. *(Miao et al., 2020)* Aside from culture conditions, the documentation of organoid growth can be done through light microscopy at key stages, including budding, post-subculturing and before the generation of frozen stocks. *(Dossena et al., 2020)* The duration of how long organoids are kept in culture could also impact cellular phenotypes, shaping cell function and molecular profiles.

Human tissue cryopreservation has been shown to increase the value of the samples obtained, and therefore, the organoids generated. *(Bui et al., 2020) (Walsh et al., 2016)* Cryopreservation is achieved by transferring tissue samples into a freezing medium, where the temperature is reduced to -80 degrees Celsius, before transferring the sample into liquid nitrogen storage. *(Han et al., 2017)* Studies have demonstrated that up to 80% of cryopreserved gut biopsy samples can support viable organoid lines. *(Konnikova et al., 2018) (Tsai et al., 2018)* However, one limitation remains the lack of research on the potential impact that the freeze-thaw cycle and long-term storage can have on the cellular function of preserved cultures.

Finally, molecular profiling supports the future value of cultures, especially in cases of translational research studies. For instance, generating genetic profiles can help subclassify organoids and thereby organise potential treatments. *(Yao et al., 2020) (De Angelis et al., 2018)* Other molecular

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profiles apart from genetic classification pose challenges, since they are more likely to change during in vivo culturing, thereby making it difficult to identify correlations with disease or treatment response. Molecular signatures, such as DNA methylation, could prove useful, considering they have demonstrated a retention of age and disease-specific alterations in culture. *(Lewis et al., 2019)*

Therefore, the above features clearly demonstrate how the lack of proper organisation or organoids stored in biobanks could prove damaging to the outcomes and validity of research studies done on them, considering their role in shaping morphology and processes within the model.

Microphysiological Systems and Organ-on-Chip Platforms

Organ-on-a-chip (OoC) technology emerged from the limitations of past methods for generating 3D models of organs. OoC microfluidic systems are micro-bioreactors constantly supplied with a given culture media for the in vitro culture of an organ at millimetre scale. *(Zhang et al., 2024)* Microfluidic technologies leverage concepts such as fluid flow, mechanical forces and oxygen gradients, helping enhance the reproducibility of human system physiology. It therefore supports the long term sustenance of various cultures, including those between the microbiota and human cells in the gut, and between brain cells and the BBB. *(Brassard & Lutolf, 2019) (Fu et al., 2020)* Organs on a chip present various advantages, including spatial orientation for interactions between cells, exposure to naturally present physical factors such as strain and fluid flow, and the exchange of nutrients and metabolites. Additionally, their miniature size means they require fewer reagents and can be more easily integrated with electronic devices for the measurement of key parameters. *(Bhatia & Ingber, 2014) (Ingber, 2016)* Finally, they reduce the cost of production per organoid and eliminate the scope for ethical concerns otherwise seen in animal models.

Microfluidic Modelling of Intestinal Environment

In recent years, gut-on-a-chip technology has developed for the study of basic gut functions. Primarily, such models are formed using microfluidic microarrays and transwell systems, including detailed methods for the creation of the end organoid devices. *(Shin & Kim, 2022)* One such model, an immunoreactive microbiota-gut axis chip platform, was developed with the purpose of studying interactions between the gut and the immune system. The platform replicated the morphology of intestinal microflora, and was able to mimic the anaerobic conditions seen in the human intestinal microenvironment due to the presence of an oxygen gradient. A reactive extracellular matrix (ECM) was added, carrying various immunomodulating mediators. The study saw inflammation produced by lipopolysaccharides causing changes in the microbiome, which further led to a proliferation in *Bifidobacterium longum*. This showcased the role of the microbiota in improving immunity of the gastrointestinal tract, and supporting the fight against infectious diseases through the release of mediators. *(De Gregorio et al., 2022)*

An innovative intestine-on-a-chip was created to replicate the oxygen gradients present between the co-culture of intestinal cells and aerobic and anaerobic gut microbes. This allowed for the growth of the human gut microbiome culture under anaerobic conditions, alongside maintaining contact with the intestinal epithelium, supporting an accurate model of in vivo conditions. *(Jalili-Firoozinezhad et al., 2019)* Another method created homeostatic mini-intestines, using the self-organisation property of cells and a scaffold to ensure the organoid forms the right structure (with the help of cues from the outlined structure provided by the scaffold). The result was an organoid in the form of tubular epithelia, containing crypt- and villi-like domains along with an open lumen that assisted the flow of fluids. *(Nikolaev et al., 2020)*

However, aside from chips solely produced on the gut microbiome, the integration of other organs is critical to accurately replicate in vivo systems and responses. One such example was a gut-liver OoC system to study intestinal permeability and metabolism, both as individual phenomena and as interacting factors impacting the other. The study used Caco2 and HT29 cells in a co-culture to replicate the intestinal epithelium, alongside primary hepatocytes isolated from a single donor in

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a separate compartment representing the liver. The system was used to understand the conversion of mycophenolate mofetil, an immunosuppressive medicine, to the active drug mycophenolic acid, and its successive metabolism to a specific metabolite. *(Milani et al., 2022)* This example clearly highlights the importance of gut-on-a-chip technologies, not only in illustrating biological processes in the gut, but also interactions between various organs closely tied to the gut.

Brain-on-Chip Systems for Neurodegenerative Research

Organ-on-a-chip technology has proven to be a critical tool in enabling the survival and development of tissue, supporting the study of cell migration and the transmission of signals. *(Raimondi et al., 2020)* This has translated into an improved appreciation of the pathology of degenerative diseases, brain tumors and various other disorders linked to the brain. *(Parker et al., 2022) (Sullivan et al., 2023) (Tang et al., 2020)*

One group aimed to study the role of BBB disruption in Parkinson's disease, caused by the accumulation of alpha-synuclein (aSyn). They formulated a brain chip using OoC technology to recreate the substantia nigra region of the brain, which has been distinctly found to be affected by the disease. The chip included a diverse range of iPSC-brain cell types, including astrocytes, pericytes, endothelial cells, microglia and dopamine neurons, enabling a replication of the vascular-neuronal interface. This platform was then used to identify and validate the efficacy of different drug targets as treatments for Parkinson's disease. A key outcome of the study was the observation that every type of stem cell was involved in the neural repair, specifically through endogenous repair by resident neural stem cells as compared to direct replacement by exogenous cells. This thereby described how restoration of brain function was achieved through the restoration of structural and functional integrity, as compared to neuronal regeneration. *(Pediaditakis et al., 2021)*

A key advantage to the application of organ-on-a-chip technology is the ability to accurately reflect the dynamic conditions seen in the body. For instance, it has been shown that neurospheres, clusters of differentiating neural cells in in vitro cultures, grown in such environments develop more elaborate neuronal networks than those raised in static conditions. Dynamic conditions involve the use of a slow, diffusion-dominant flow that enables the continuous supply of nutrients and oxygen, along with the movement of cytokines and the removal of metabolic waste products present in the given part. In fact, the inclusion of an extracellular matrix microfluidic device was shown to improve the maturation of brain organoids. *(Cho et al., 2021)* This evidently demonstrates the importance of a dynamic environment for the growth of human brain organoids accurately reflecting the real brain.

One group formulated the plans for a microfluidic chip to simulate flow conditions in the brain, with the help of neurospheroids, self-assembling clusters of neural cells. These spheroids rely on the ability of small groups of cells to form polar structures without the assistance of any pattern or other material. Possessing the ability to capture complex cell-cell interactions and operate at a higher functionality, these neurospheroids are powerful biological platforms for in vitro models. *(Dingle et al., 2015) (Paşca et al., 2015)* The chip was created using a printing process, soft lithography, to bond polydimethylsiloxane (PDMS) microfluidic chambers together. The top part of the device contained the culture medium, which, with the help of a micro-pumping system, flowed to the neurospheroids in the bottom part through osmosis. The purpose of the microfluidic chip was to test the toxicity of amyloid beta protein present in Alzheimer's Disease. The outcome of the study demonstrated that interstitial flow impacts the size distribution of neurospheroids, and ultimately increases toxicity of amyloid beta protein as compared to static conditions. *(Park et al., 2015)*

Another study used the OoC technology to mimic the process of human pluripotent stem cell differentiation into neuronal and astroglial cells. The purpose of the study was to measure migration of the neural cells with respect to chemotactic cues (the differences in chemical substance concentrations). The device used contained three PDMS layers attached to a glass surface for support. The first sheet depicted the vascular component in the form of blood vessel lumens, along with pillars to prevent a collapse; the second sheet was made of a porous membrane to model the BBB; finally, the third sheet represented the neuronal compartment, containing neural tissue. The top and bottom

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compartments had four perfusion channels (two inlets and two outlets) to support dynamic conditions through the flow of given fluids. *(Kilic et al., 2016)*

Certain studies have also stipulated the integration of electrodes into OoC technology, enabling the study of burst-firing power, measuring the strength of neuron action potential emissions, and their frequency. Further, removable inserts could support the compartmentalisation of neurons from different brain areas, while still enabling processes and interactions between these cell populations when removed. This concept, while still lacking perfusion, overcomes various challenges seen in static conditions, including the lack of control over the integration of different compartments, offering a potential alternative for certain inquiries. *(Soscia et al., 2017)*

The inclusion of the BBB within a single brain OoC model was a critical step towards miniaturising in vitro models for increased applicability in drug testing. One group achieved this by first filling the brain chamber with collagen hydrogel, a gel-like material. Within it, they embedded three cell types: N2a neuroblastoma cells, a type of cancer cell used as a neuron model; C8D1A immortalised astrocytes, cells that protect neurons; and BC-2 immortalised microglia, the immune cells of the brain. Once the liquid had reached a gel-like state, the BBB compartment was defined by plating bEnd.3 (brain endothelial-3) endothelial cells onto the hydrogel. Finally, the use of a rocker shaker, an instrument that agitates liquids back and forth, ensured the recapitulation of fluid force and flow. *(Koo et al., 2018)*

Reconstruction of BBB Function in Microfluidic Platforms

Various models have been conceptualised and crafted in an attempt to replicate the basic features of the blood-brain barrier. Such in vitro structures are critical in forming an understanding of the movement of molecules between the barrier, changes seen during disease, and ultimately, key processes leading to neurodegeneration.

Various types of models have been experimented with in the case of the BBB. Primary cultures from brain endothelial cells would be the ideal option in the case of drug development; however, challenges with availability render them ineffective for widespread applications. Mouse or rat cell populations, while easier to acquire, pose the challenge of low yield, making their frequent use an impractical alternative. *(Jiang et al., 2019)* Another model type is composed of self-renewable cells, such as pluripotent stem cells, which can differentiate into somatic cells. Such models have demonstrated their worth in achieving an approximation of the physiological nature of the barrier; however, they become limited in replicating changes seen in key features, such as permeability, seen in pathological states. *(Raimondi et al., 2020)*

Static models are composed of two chambers, dividing the vascular compartment from the working tissue (parenchymal) section of the organoid. Such models are separated by a semipermeable membrane, acting as the physical barrier seen in the human body as the BBB. *(Bors & Erdő, 2019)* Contrasting these are dynamic models, which are enhanced alternatives to conventional 2D systems. They pose the challenge of higher flow rate requirements compared to in vivo conditions due to the larger size of microtubes in organoid models. *(Raimondi et al., 2020)*

Considering the various challenges in the above model types, microfluidic structures of the BBB arose. Various groups have tackled the challenges of replicating its complex physical features and functions in different ways. One adopted approach was the creation of a neurovascular unit with four parallel PDMS channels: the first contained the medium; the second held cortical neurons in a given collagen hydrogel; the third carried primary astrocytes in a different collagen hydrogel; and the fourth kept endothelial cells. Each channel could communicate with those adjacent to it, enabling signaling and contact between cells as seen in the human body. *(Adriani et al., 2017)*

Another aspect to be addressed was the need to invert the model during its assembly to ensure proper mimicry of the BBB's two compartments. In the body, the BBB separates the vascular side, formed by brain endothelial cells, from the parenchymal face, holding the functional cells of the brain. Since the membrane is traditionally placed horizontally within the device, there is the tendency, due to gravitational forces, for cells in the upper compartment to settle on the membrane while ones

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underneath do not. One research group devised a unique solution to this. They created a device containing three PDMS layers, with a polycarbonate membrane separating the first sheet from the others. The first PDMS layer held microvascular endothelial cells from the human brain, while the other two layers held astrocytes and pericytes. By turning the device upside down after twelve days, the group ensured both segments of the device were plated appropriately on the membrane. Further, a flippable backpack was included to avoid the overturning of inlet and outlet tanks during the inversion of the device. *(Brown et al., 2015)*

One group created a BBB microfluidic model to study its function along with drug delivery. The device included PDMS sheets, which created perfusion channels; glass electrode layers that allowed for appropriate monitoring of the barrier's integrity (TEER); and a polycarbonate membrane to separate the luminal (blood vessel) and abluminal (brain tissue) compartments. High stress in the abluminal channel recapture the forces felt by endothelial cells in blood vessels. The luminal channel, on the other hand, experiences low stress, replicating the environment of astrocytes which are subject to less fluid flow. The electrodes use a four-point sensing structure with the help of a pair of two AgCl electrodes. This structure helped reduce the role of wiring resistance, enabling greater accuracy in measured values. However, monitoring done by electrodes remained limited since it covered 75% of the culture surface. *(Booth & Kim, 2012)*

A given study focused on eliminating cross-species incompatibility to generate results that were physiologically relevant. It achieved this by culturing human brain cells, namely pericytes, astrocytes and iPS-endothelial cells, which were then embedded as a co-culture into channels on a chip. The chip also contained ports filled with a fibrin hydrogel, which acted as a kind of 3D extracellular matrix scaffold, providing support in a way similar to the natural brain environment. The results of the study indicated that this process had yielded a physiologically relevant structure which could be used in a variety of applications. *(Campisi et al., 2018)*

One of the most important properties of the BBB is its permeability, which can be measured using experimental means, such as hydrophilic tracers. *(Banerjee et al., 2016)* Trans-Endothelial Electrical Resistance (TEER) measures the electrical resistance across a surface of endothelial cells, and has been shown to correlate with permeability. In this way, high TEER values reflect a more tight barrier, and therefore, lower permeability, while low TEER values indicate a compromised barrier with greater permeability. *(Helms et al., 2016)* Other methods of assessing BBB properties include transport studies with sample molecules and tight junction immunostaining. *(Butt et al., 1990)*

Reconstructing the Gut-Brain Axis on a Chip

A microfluidic system combining the gut and the brain is critical in providing a comprehensive simulation of the Gut-Brain Axis (GBA). *(Kim et al., 2021)* Such a device would be able to replicate the bidirectional communication and interaction between the true, supporting an improved approach to studying disease and pathology. *(Chen, Ding, et al., 2023)*

One of the most common methods for achieving a GBA system in vitro is through a co-culture of intestinal epithelial cells and brain endothelial cells. This can be further supported by mimicry of the BBB through microfluidic channels that support fluid flow. Researchers of such a study found that known microbial byproduct-mediated interactions between the gut and the BBB yielded similar responses from the microfluidic chip. *(Kim et al., 2021)*

A unique alternative to such a chip was the use of a petri dish to host a gut-brain system. Here, separate components for the mini-gut, composed of endothelial cells, and mini-brain, created with crayfish nerves, were combined with a microporous cell culture membrane. This membrane helped identify key molecules and signalling pathways through which the two organs would communicate. The use of crayfish nerves were appropriate for such a case given they mirrored the electrophysiological responses seen in animal studies. The two chambers in the petri dish were also allowed a fluid connection, enabling the movement of molecules that could be monitored. The study was able to identify serotonin, a neurotransmitter, as being a key signalling molecule. *(Chapin et al., 2020)*

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The role of metabolites from gut microbes and extracellular vesicles on neurodevelopment and neurodegeneration was of key interest to one group. They used human iPSCs to create a microfluidic chip of the gut-brain axis, experimenting with the impact of microbiota-derived metabolites and exosomes. The study found that the movement of these components through the axis had the potential to trigger neural differentiation along with enhancing the expression of synapse-linked proteins. *(Na Yeon Kim et al., 2024)*

Advanced Multi-Organ Architectures

Aside from microfluidic systems solely integrating the gut and the brain, other organs have been included to enable a study of either a specific application or an interaction between the given organ and the GBA. For instance, researchers formulated a gut-liver-brain simulation using a microphysiological system (MPS). The system connected brain cells to in vitro colon and liver tissue models, supporting the study of immune cells and nutrients that flowed through channels between these three elements. *(Trapecar et al., 2021)* This highlights a prime example of how multiple organs can be bridged in such a system to enable an investigation on interactions between them.

In 2017, the European Research Council funded their first project on a bionic platform, termed 'MINERVA'. The platform was developed to examine the role of the microbiota in neurodegenerative diseases, namely Alzheimer's. *(Raimondi et al., 2019) (Loh et al., 2024)* The main challenge of such a system is the massive complexity, integrating five microfluidic organs-on-a-chip to form the final platform. The project modelled the microbiota, gut, immune, BBB and brain, with each element forming a separate compartment. The microbiota was formed using a hydrogel matrix that mimicked the intestinal mucus. The matrix hosted the gut microbiota with the help of fecal samples from healthy donors and Alzheimer's patients. The gut was replicated using gut epithelial cells plated on a microporous membrane. The immune device contained lymphocytes and macrophages, with a membrane preventing their exit. The BBB section held endothelial cells and astrocytes laid on either side of the microporous membrane. Finally, the brain compartment aimed to replicate the extra-cellular matrix, encompassing neurons, astrocytes and microglia. The microporous membrane divides each device into parts, preventing the movement of cells to the next device along with the flow of the culture medium. *(Alessandro Marturano-Kruik et al., 2018) (Tunesi et al., 2016)* This platform represents a massive step in the direction of multi-organ chips, enabling the possibility of a wider range of studies and corresponding conclusions for both a physiological understanding and improved approaches to treating disease.

Applications in Drug Discovery and Toxicology

Drug development studies have been a critical space in medical research. Up until recently, animal models have been critical in this respect, to support the understanding of molecular mechanisms at the center of various diseases. However, such reproduction of in vivo set-ups pose various challenges, including high costs, ethical complications, problems with replicating patient genetics and the inability to translate animal to human. *(Dawson et al., 2018) (Jucker, 2010)*

This has set the stage for the growth of human iPSC technology, which provides answers to the stated concerns. The ability to generate models from all patient genetic backgrounds along with the possibility of differentiating these lines into almost any cell type, make the field of high interest to groups across disease focuses. The technology has been leveraged in cases of relatively simple 2D cell cultures, which remain limited in the recreation of the physiological environment but provide an affordable, highly reproducible method. *(Argentati et al., 2020) (Ho et al., 2018)* Human iPSCs have also been used to create a 3D matrix that replicates cell-cell interactions and structural frameworks as seen in the natural world. *(Centeno et al., 2018)* Finally, the gap created by the absence of fluid dynamics between cells and organs has been filled with organ-on-a-chip technology, a novel approach to integrate organoids into a dynamic system that recapitulates flow conditions in the body. The genetic makeup of patients also impacts drug response, to the extent of causing unwanted side effects. This means patient-derived iPSC models can be effective in such cases, since they support a

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personalised prediction of the safety of different drugs for the given individual. *(Jodat et al., 2019) (Inoue & Yamanaka, 2011)*

Between 2002 and 2012, only 1 out of 244 (0.4% of) tested compounds for Alzheimer's in clinical trials were approved. *(Murray et al., 2014)* Other neurodegenerative diseases, such as Parkinson's and amyotrophic lateral sclerosis, also have similarly low success rates. *(Bolognesi, 2017) (Boucherie et al., 2021)* This is a result of the lack of knowledge of the mechanisms and processes causing disease, along with variability between individuals. For instance, the efficacy of donepezil, the first approved drug in AD treatments, remains highly variable, as its effects are closely linked to genetic aspects of patients. *(Lu et al., 2020)*

Within OoCs, iPSCs can grow in 2D conditions as a monolayer or in 3D matrices, where they are embedded inside such moulds. *(Fanizza et al., 2022)* Microfluidic devices provide mechanical and chemical stimuli, ensuring the creation of a dynamic environment that influences cell interactions. Aside from the benefits in terms of greater accuracy to the human body, such methods also help the 3Rs paradigm (Reduce, Replace, Refine) in the context of in vivo animal testing. *(Kirschner, 2021)*

Drugs that are orally ingested are absorbed by the intestine, metabolised by the liver, transported via blood circulation to the given target organs, and finally removed from the body by the kidneys. Therefore, the modelling of the gut, liver, kidney, heart and lungs are critical alongside the inclusion of the BBB and a brain-on-a-chip in applications linked to drug testing. Vascularisation also becomes even more critical when it comes to OoC models, especially since drug transport occurs through the bloodstream to tissues of the organ. *(Osaki et al., 2018)*

OoC Devices for Drug Screening

Liver

The liver plays a critical role in drug metabolism and detoxification, and is therefore the primary target for drug-induced toxicity testing. Groups have worked with livers-on-a-chip to investigate hepatotoxicity induced by novel drugs. The study involved the replacement of primary human hepatocytes (PHH) with iPSC-derived hepatocytes, the main liver cells. It concluded that the drug effects in PHH were more evident than in iPSC-HEPs. *(Rennert et al., 2015) (Vernetti et al., 2015)*

Kidney

Nephrotoxicity caused by drugs is the second cause for the failure of drugs. The excretion process for drugs involves filtration by the glomerulus, secretion and reabsorption by the collecting duct. While the complexity of the kidney's structure cannot be replicated in the lab, research groups have focused on the development of a glomerulus-on-a-chip. *(Sakolish et al., 2019) (Jang et al., 2013)* Chips developed so far lack functional renal podocytes, specialised epithelial cells found in the glomerulus. These cells are necessary in forming the filtration barrier which enables selective permeability. *(Greka & Mundel, 2012)*

Heart

The availability of iPSC-derived cardiomyocytes led to the conception of novel methods for modelling disease to enable effective drug testing. However, they are primarily limited by the lack of understanding of cellular interactions within the heart tissue, lowering their effectiveness as culture systems. *(Fanizza et al., 2022)*

Gut

Oral administration is a preferred route of drug delivery, making the gut a necessary element for drug screening studies. *(Jain, 2008)* Intestinal models have the potential to accelerate the pace of research studies focused on the absorption, metabolism and toxicity of drugs. *(Beaurivage et al., 2019)* They also enable the modelling of intestinal disease, such as inflammatory bowel disease, which have

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been shown to play a role, via gut dysbiosis, in brain conditions and function. For instance, one group created a drug screening organoid platform with 304 microchannel wells, with each being capable of culturing an organoid simultaneously on a chip. This supported a high-throughput model that could evaluate multiple drugs or different concentrations of the same drug in an extremely efficient manner. *(Zhu et al., 2024)*

Multi-Organ

One study aimed to create a multi-organoid microfluidic platform with a capacity for up to six different organoids. The set-up used 96-well round-bottom plates to culture liver, heart, lung, testis, colon and brain organoids. When combined into a microfluidic platform, this resulted in a six-tissue microphysiological system. The key finding from the study was the ability for liver organoids to metabolise capecitabine and cyclophosphamide, leading to downstream drug toxicity, or the drugs' interaction with unintended pathways, in other organoids. *(Skardal et al., 2020)* These two examples reflect the potential for intestinal organoid systems to impact vast fields in the domain of research and medicine, with an even greater magnitude of influence as the technology improves.

Stem-Cell-Based Neural Organoids

The complexity of the brain makes it one of the most difficult organs to replicate in the lab, especially in cases of drug analysis where it becomes critical to reproduce the in vivo environment with as much integrity as possible. Research groups over the years have adopted various methods and approaches to modelling the brain to support drug testing in the case of neurodegenerative diseases. This section focuses on those efforts which used patient-iPSCs to study various diseases, supporting the process of drug screening. *(Yan et al., 2018) (Raja et al., 2016)*

One study used an AD iPSC-based 3D culture of neurons and glial cells, demonstrating a rise in key markers, including pathological Beta-amyloid, oxidative stress and Tau. *(Rouleau et al., 2020)* Results from the study were clear evidence of the potential of 3D models to replicate key features of neurodegenerative disease. Nevertheless, it did also highlight key limitations seen in such structures, namely the lack of reproducibility and the absence of vascularisation, critical in the case of drug testing for understanding drug distribution. *(Bhise et al., 2014)* These challenges will emerge in future studies as well as a prominent gap between in vivo and in vitro set-ups.

Multicompartment microfluidic devices have also been developed for the study of the pathological basis of key neurodegenerative diseases. One such device included a central chamber connected to four outer chambers through microgrooves. *(Fantuzzo et al., 2017)* This helped separate different neurons, namely excitatory (glutamatergic), inhibitory (GABAergic) and dopaminergic types. Another approach involves the creation of a neurovascular unit-on-a-chip. Here, the device would contain vascular and brain components, inhabited by constituent elements such as astrocytes, pericytes, microvasculature endothelial cells and collagen gel. *(Brown et al., 2015)* Finally, personalised medicine is another aspect to such microfluidic platforms. One research group taking this approach filled parallel culture channels with iPSCs. The two channels had fixed boundaries around them, formed by three medium channels. The culture and medium compartments were also separated by a micropillar array, supporting the movement of nutrients and waste to and from the cell. *(Wang et al., 2018)* By using human iPSCs, the group could form patient-specific models to support the study of a given individual's brain environment and drug response.

The trisynaptic circuit of the hippocampus is a critical connection in the brain, especially for studying neurodegenerative diseases impacting this brain region, including Schizophrenia, AD and PD. *(Lieberman et al., 2018) (DeTure & Dickson, 2019) (Regensburger et al., 2014)* Other studies of network pathways identified cytochrome P450 pathways as most affected by lipopolysaccharides and cytokines cocktail treatment. *(Kim et al., 2016)* Such a conclusion was critical since the cytochrome P450 pathways are critical in drug metabolism in the brain *(Miksys & Tyndale, 2013)* and disease response *(Navarro-Mabarak et al., 2018)*.

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Parkinson's Disease is one of the most significant neurodegenerative diseases, with data suggesting a massive prevalence; as of 2021 reports, nearly 12 million people worldwide had PD. *(Luo et al., 2025)* A key circuit impacted by the disease is the nigrostriatal pathway. To further understand this, one research group created a microfluidic platform to mimic the circuitry of the pathway, with the aim of understanding the mitochondrial impairment caused by the OPA1 mutation in Parkinson's patients. To achieve this, patient-derived iPSCs were first differentiated into dopaminergic and striatal medium spiny neurons, each placed in a separate compartment. A central chamber connected the two side chambers via microgrooves. The study concluded that the OPA1 mutation in PD patients led to a non-standard morphology and a loss of mitochondria. This was further linked to the decline in functional synapses seen in neurodegenerative diseases like Parkinson's. *(Iannielli et al., 2019)*

Through data from the 2019 Global Burden of Disease (GBD) database, we have been able to study the global picture of Alzheimer's Disease. Since 1990, the incidence and prevalence of AD has increased by nearly 150%, a staggering number demonstrating the growing demands of a cure. *(Li et al., 2022)* Similar to other disorders, key hallmarks of Alzheimer's Disease have been identified, including neurofibrillary tangles and amyloid-Beta plaques; these have been hypothesized to contribute to the death of neurons. *(Eckerström et al., 2018)* Mapranosis (or Microbiota Associated Proteopathy and Neuroinflammation) describes the process through which amyloid proteins, produced by microbes, increase the misfolding of amyloid-Beta structures in the brain, and thereby augment inflammation across the nervous system. *(Sharon et al., 2016) (Uesaka et al., 2016)* Another primary hallmark of the disease is the abnormal nature of the Tau protein. A study investigated the cellular uptake and axonal transport of Tau monomers and oligomers. It found that aggregates of Tau protein, present as oligomers, led to the misfold and aggregation of normal Tau proteins. In this way, it was able to demonstrate the spread of pathology. *(Usenovic et al., 2015)* The absence of microglia, cells that are critical in the mediation of immune responses in the brain, represents yet another key feature characterising AD. *(Sabogal-Guáqueta et al., 2020)* Microglial cells formed from iPSCs have demonstrated a level of integrity in recapturing in vivo characteristics. *(Abud et al., 2017)* Models using such cells were able to demonstrate that microglia activation and recruitment was caused by amyloid-Beta, linking two prominent features of AD. *(Park et al., 2018)*

Limitations, Opportunities and Pathways Forward

Current Landscape

Most of the information known today about the microbiota-gut-brain axis (MGBA) has come from studies on mice in vivo. This causes three primary problems: 1) animal communication pathways differ from humans; 2) high variability with difficulties in singling out an individual parameter impacting the gut and the brain; 3) economic and ethical problems associated with such studies. *(Dobson et al., 2019)* Such challenges have led to the implementation of the 3R's principle associated with animal studies: replacement (with approaches that do not use animals), reduction (lower number of animals used for the same study), and refinement (modifying procedures to minimise animal suffering). *(European Commission, n.d.)*

To solve this issue, in vitro models were introduced, with the aim of recapturing disease mechanisms, with the ability to easily isolate different parameters. 2D cultures represent a cost-effective solution, with a level of robustness applicable to cell types along with simplicity in handling them. However, cells in our body are surrounded by their unique 3D matrix, exposing them to signals and stimuli that are absent in 2D structures. Another class of such models are assembloids, which depend on the controlled formulation of 3D cultures to appropriately recapture cell-cell interactions. However, such assembloids are still a long way away from perfect, considering they need to modulate dynamic features seen in vivo, and ensure the viability of cells in larger constructs. *(Pașca, 2018)*

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OoC technology supports cell culturing in individual chambers, along with microfluidic channels to ensure the microenvironment's distribution is managed. Such set-ups enable complex conditions, including the formation of gradients, the flow of mediums for the exchange of molecules, cell differentiation, and operating parameters, each of which contribute to the overall recapturing of the target part's physiology. *(Bhatia & Ingber, 2014) (Ingber, 2016)* Advancements in miniaturisation have further reduced the load of resources for such models, while sensors allow for continuous monitoring of key parameters, some of whom have been described earlier. Finally, connections to multi-organ platforms using microfluidic technologies in organ chips guarantee a heightened level of comprehension on the underlying biological mechanisms of organ pathology. *(Jalili-Firoozinezhad et al., 2019)*

Challenges

The space of organoid development remains blotted with countless obstacles. In summary, the primary concerns, similar to those raised in the previous section, remain: the lack of a complete and comprehensive representation of the cellular diversity and architecture of organs *(Vargas-Valderrama et al., 2020) (Múnera et al., 2023)*; the absence of critical cells, including vasculature, which impacts disease model accuracy *(Koike et al., 2019)*; and finally, the inability to standardise, resulting in experimental inconsistencies as a result of this variability. *(Fleischer et al., 2020)*

A preliminary requirement for effective modelling of organs is the inclusion of a blood-like flow. The absence of vascularisation in most organoid systems continues to act as a primary barrier for effective replication of in vivo environments. *(Zhang et al., 2023)* Since most organoid set-ups are static in nature, as compared to their fluid state in the human body, nutrient and oxygen delivery is limited in vitro. Vascular structures are also critical for the mimicry of drug exchanges taking place between the blood and parenchyma of organs. *(Pollet & den Toonder, 2020)* Vascularisation of organoids also extends to the case of drug screening applications, since it demonstrates how drugs are distributed across the body once ingested.

A major challenge is the lack of accurate representation of in vivo structures and functions. Taking the case of intestinal organoids, while basic versions have improved our understanding of intestinal biology and disease, the failure of replicating a highly complex, intricate gut environment poses a key limitation. *(Günther et al., 2022)* Ideal intestinal models would include a wider range of bacterial species, alongside other components of the gut that are less commonly studied, such as viruses and fungi. A larger challenge with simplistic models is their tendency to exaggerate the role played by specific microbiota, especially in studies involving individual microbial effects. *(Puschhof et al., 2021)* The difference between in vitro model sizes and densities also causes discrepancies in the replication of in vivo conditions. Finally, even after incorporating multiple cell types in a model, the lack of advanced imaging and sequencing prevents the appropriate depiction of the complex interactions existing between them, preventing researchers from capturing a complete picture. *(Zheng et al., 2024)*

Another key gap is the limited incorporation of linked cell types directly connected between the given organ and another system. For instance, gut microbiome studies must include neural and vascular cells, and vice versa, to ensure a complete representation of the interplay seen between GBA components. Replicating the immune cell environment is also necessary in most disease studies, given that while the organ may not be a part of the immune system, disease would still cause an immune response. Therefore, incorporating human immune cells improves the modelling of such interactions in disease-specific studies. *(Geller et al., 2017)*

Taking this a step further, the need for multiple organs-on-a-chip has long been established as a key requirement, considering the interconnected processes, pathways and elements between body systems and parts. Going back to the drug screening application, the processes of drug absorption, distribution, metabolism and excretion (ADME) must be integrated. This demands the development of all organs responsible for such processes to be incorporated alongside the target organ, posing

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countless further challenges and a massive leap in complexity of the overall framework. *(Kimura et al., 2018)*

The culture media is a critical element of organoid set-ups, since it represents the source of nutrition for all cell types. Hence, the optimisation of a common culture media is necessary; while it can be achieved with relative ease in a few connected organs-on-a-chip, platforms with more than three organs make the task increasingly difficult. While a serum-free medium, to eliminate the risks of inter-batch biological variability, could be a solution, it has yet to be tested on a wider range of cell types. *(Stein, 2007)* Closely tied to this is the use of PDMS in microfluidic organoids. As a material that forms the channels and membranes of OoCs, a fatal property of PDMS in such a use case is its tendency to absorb small hydrophobic molecules. This means hydrophobic drugs are absorbed into the PDMS walls, thereby lowering the actual concentration and rendering the end results inaccurate. *(Toepke & Beebe, 2006)*

The long-term viability and functionality of the co-cultured cells also exists at a rudimentary level currently. This heavily restricts the application of such organoids, especially in critical cases such as chronic modelling and drug testing. One reason behind this is the lack of vasculature, preventing the delivery of nutrients and oxygen critical for survival of cells, resulting in widespread cell death. Even if organoids can be sustainably maintained, the maintenance of physiological relevance, especially in the case of chronic immune responses, prevents the lasting of a model from yielding productive results. *(Zheng et al., 2024)*

A comprehensive strategy is yet to be formulated to ensure results from OoC-based studies are effective in assessing the safety of the first in-human dose. Ideally, pharmacokinetic models can model the mechanisms of drug actions and resultant responses in the human body. By first determining their safety in animal studies with fixed parameters, and then scaling the results for humans, a process can be constructed to ensure utmost safety in initial human trials while also not limiting the process of drug discovery with avoidable restrictions. *(Jong Hwan Sung et al., 2019)*

Finally, outside the realm of technical hurdles, in comparison to traditional cultures, 3D models are largely inaccessible for the majority of organisations and applications. This is a result of the complexity of OoC-based drug screening studies (especially when integrating the differentiation of cell types to iPSCs) *(Kimlin et al., 2013)* and the involved research costs, which together render the technology non-viable. Solutions, while still being developed, could be in the form of increased automation of instruments, especially during culturing and analysis, and the development of a high-output efficient drug screening platform. *(Fanizza et al., 2022)* Alongside these are the ethical issues associated with the development of cerebral organoids using stem cells. Not only are there challenges with consent from stem-cell providers, but also debates regarding whether such organoids are conscious, and therefore, can experience pain. While current cerebral organoids, given the absence of any sensation, are unlikely to be conscious, future developments must be done considering potential implications if advancements produce entities that are self-conscious or can access consciousness. *(Lavazza & Massimini, 2018)*

Future Directions

While the path ahead to successfully crafting a single MGBA multi-organ platform is long, interdisciplinary efforts have acted as the first steps in the right direction. For instance, bioprinting is an innovative approach that incorporates materials, cells and biological elements. Custom-made architecture, including manipulation of shape, porosity and interconnectivity, further enhances the effectiveness of such constructs. *(Paxton et al., 2017)* Hydrogels are another innovative technique to create microenvironments for cell cultures. Of greatest concern, however, is the impact that printing processes, namely the stress involved during the production of such models, can have on cell behavior. *(Moroni et al., 2018)* Epidemiological studies can also be crafted by taking stem cells of patients from different regions, helping obtain AD information from different populations. Finally, a merger between bioprinting and microfluidics technology described earlier can help develop automated devices to support drug testing and screening. *(Bhise et al., 2016)* Such efforts highlight the

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ever-evolving nature of the field, with constant development supporting an extension in its accuracy of in vivo-modelling and its applicability across a wider set of use cases.

One of the most exciting developments, while still largely on the conceptual level, have been the concept of multi-organ platforms, or a 'body-on-a-chip'. The concept aims to recreate the complete human responses, something that cannot be recapitulated by a single organ-on-a-chip. The most popular approach to crafting such a concept has been a connection of different organs-on-a-chip which supports the analysis of cross-organ communication analysis. This can be applied to cases of drug toxicity, where the modelling of all organs with the help of iPSCs from a specific patient can predict their personalised response to a given treatment along with side effects. *(Yin et al., 2021)* Such a concept could very well be the future of healthcare treatments as we know it.

Conclusion

As a complex physiological network closely tied to the disruption of biological processes in neurodegenerative diseases, the gut-brain axis has recently been adopted as a novel lens to approach neurological disorders. Through the review of existing literature describing efforts to curate various organoid and organ-on-a-chip platforms, this paper illustrates the current state of the GBA-organoids field applied to neurodegeneration. Spanning work across individual intestinal and cerebral organoids, gut-brain in vitro models, and microfluidic systems, models of disorders such as Alzheimer's and Parkinson's and their application to drug screening were evaluated. Critical limitations direct future directions of the field, including model vascularisation, accurate replication of microbiome and immune complexity, standardised protocols, comprehensive cellular and organ diversity, and functional-structural integrity to human organ systems. As next-generation complementary tools, capable of capturing elements of human-specific biology absent in animal models and other 2D cell cultures, organoids and microphysiological systems represent the next step in replicating disease morphology and identifying novel therapeutic approaches.

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