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REVIEW

Cellular interplay to 3D in vitro microphysiological disease model: cell patterning microbiota–gut–brain axis

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Abstract

The microbiota–gut–brain axis (MGBA) has emerged as a key prospect in the bidirectional communication between two major organ systems: the brain and the gut. Homeostasis between the two organ systems allows the body to function without disease, whereas dysbiosis has long-standing evidence of etiopathological conditions. The most common communication paths are the microbial release of metabolites, soluble neurotransmitters, and immune cells. However, each pathway is intertwined with a complex one. With the emergence of in vitro models and the popularity of three-dimensional (3D) cultures and Transwells, engineering has become easier for the scientific understanding of neurodegenerative diseases. This paper briefly retraces the possible communication pathways between the gut microbiome and the brain. It further elaborates on three major diseases: autism spectrum disorder, Parkinson's disease, and Alzheimer's disease, which are prevalent in children and the elderly. These diseases also decrease patients' quality of life. Hence, understanding them more deeply with respect to current advances in in vitro modeling is crucial for understanding the diseases. Remodeling of MGBA in the laboratory uses many molecular technologies and biomaterial advances. Spheroids and organoids provide a more realistic picture of the cell and tissue structure than monolayers. Combining them with the Transwell system offers the advantage of compartmentalizing the two systems (apical and basal) while allowing physical and chemical cues between them. Cutting-edge technologies, such as bioprinting and microfluidic chips, might be the future of in vitro modeling, as they provide dynamicity.

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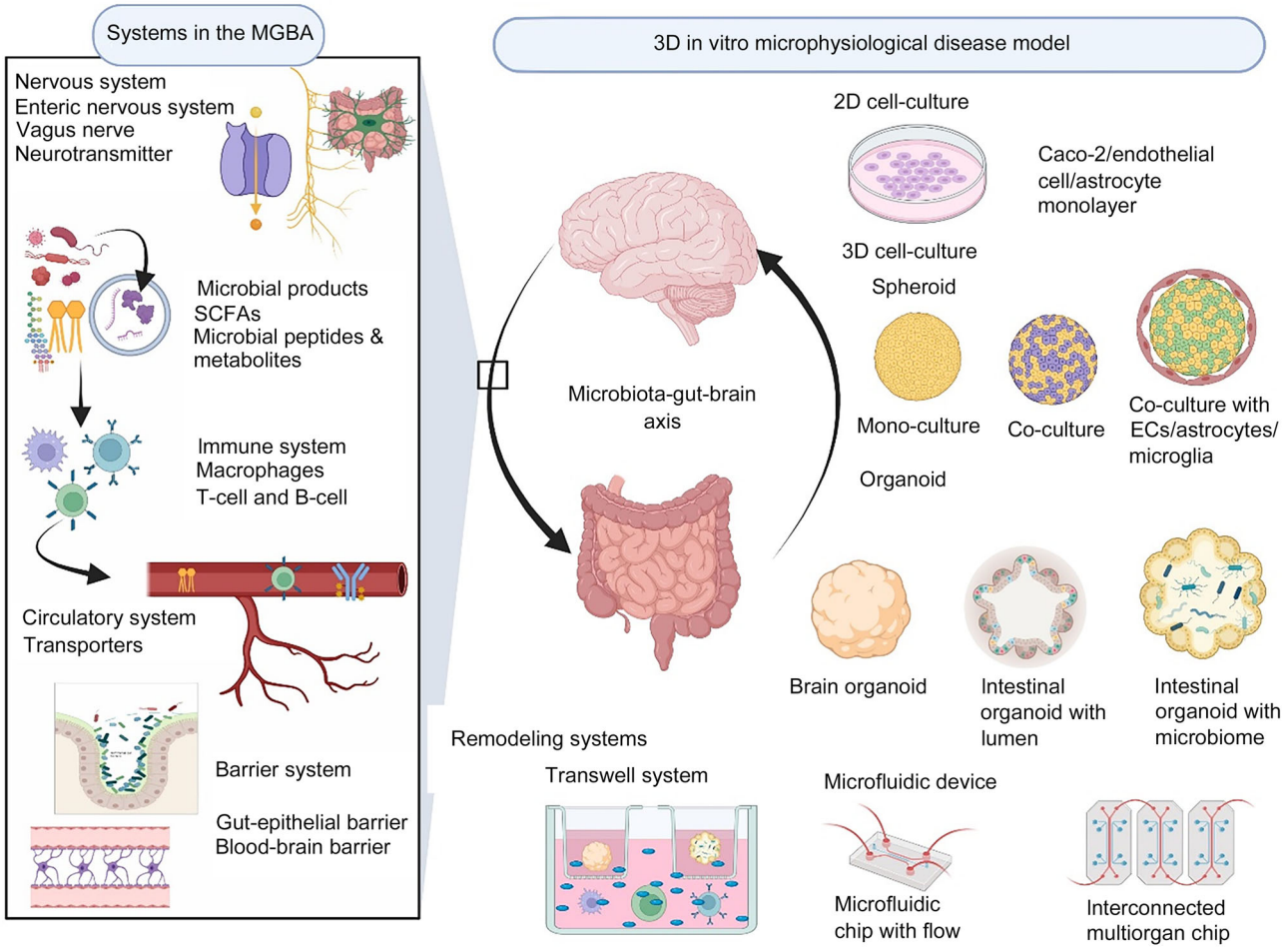
Graphic abstract



Keywords Microbiota–gut–brain axis · Neurodegeneration · 3D disease model · Organoid · Transwell system

Abbreviations

3D	Three-dimensional
5-HT	5-Hydroxytryptamine
AD	Alzheimer's disease
ADHD	Attention-deficit hyperactivity disorder
ALS	Amyotrophic lateral sclerosis
ANS	Autonomic nervous system
APP	Amyloid precursor protein
ASD	Autism spectrum disorder
BBB	Blood–brain barrier
BCS	Biopharmaceutical classification system
BM	Basement membrane
BMECs	Brain microvascular endothelial cells
BTBR	Black and Tan Brachyury
Caco-2	Cancer coli-2 cell line
CNS	Central nervous system
CSF	Cerebrospinal fluid
CYP	Cytochrome P450
DCs	Dendritic cells
ECs	Endothelial cells
ECCs	Enterochromaffin cells
EECs	Enteroendocrine cells
ENS	Enteric nervous system
FMT	Fecal microbiota transplant
GABA	γ-Aminobutyric acid
GI	Gastrointestinal
HDAC	Histone deacetylase
hESCs	Human embryonic stem cells
hNPCs	Human neural progenitor cells
HT-29-MTX	Human colorectal adenocarcinoma cell line
IFN	Interferon
IL	Interleukin
iPSCs	Induced pluripotent stem cells

KP	Kynurenine pathway
LPS	Lipopolysaccharide
LR	*Lactobacillus reuteri*
MGBA	Microbiota–gut–brain axis
MTX	Methotrexate
NDDs	Neurodegenerative diseases
NE	Norepinephrine
NMR	Nuclear magnetic resonance
PCs	Pericytes
PD	Parkinson's disease
PDMS	Polydimethylsiloxane
PNS	Peripheral nervous system
SCFA	Short-chain fatty acid
SPF	Specific pathogen-free
T84	Transplantable human carcinoma cell line
TEER or TER	Transepithelial/transendothelial electrical resistance
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-α
TPH	Tryptophan hydroxylase
VN	Vagus nerve
ZO-1	Zonula occludens-1

Introduction

Over the years, clinical and preclinical studies have established the possibility of bidirectional exchange between the gut and other organs, including the brain [1]. The intestine, the largest immune organ, is responsible for digesting important nutrients and harbors diverse microorganisms that facilitate this process. The complexity of the communication between the microbiota and the host via the gut to various organs remains unclear [2]. The microbiota–gut–brain axis (MGBA) is the direct axis used for communication between the brain and the gut microbiota. The axis indicates a bidirectional connection between the two organs. The microbiota is crucial for homeostasis and regulation of bodily functions. Dysregulation has been implicated in neurological disorders, including Alzheimer's disease (AD), Parkinson's disease (PD), autism spectrum disorder (ASD), and amyotrophic lateral sclerosis (ALS), and neural development [3]. Growing evidence, reduced genome sequencing costs, and metabolomic technology have attracted the attention of scientists. 16S amplicons and full-genome sequencing have enabled the estimation of the abundance of specific microbes. Advanced statistical tools, including principal component analysis, multidimensional scaling algorithms, and comparative relative abundance, have allowed factors in the diversity metric and provided a robust understanding of the microbiota [4, 5]. The diversity of microorganisms is large and is largely affected by the maternal microbiota at the fetal stage and later by diet, environment, antibiotic exposure, metabolism, and age as the person grows [6]. Gut microbial populations also play a role in homeostasis by maintaining bodily functions. It helps in vitamin generation, metabolism, protection against pathogens, and maintenance of the intestinal-systemic barrier [3]. It acts directly or indirectly via the vagus nerve (VN) and neural, immune, and endocrine systems by influencing neurotransmitters from microbes, such as microbial metabolites and hormones [7]. The involvement of the gut microbiota in brain signaling is confirmed by microbe-derived signaling molecules, such as short-chain fatty acids (SCFAs), tryptophan (Trp) metabolites, and secondary bile salts. These microbe-derived molecules interact via enteroendocrine cells (EECs), enterochromaffin cells (ECCs), and mucosal immune cells; however, the mechanism by which these molecules cross the intestinal barrier and gain access to systemic circulation across the blood–brain barrier (BBB) is still not understood. It is speculated that SCFAs, Trp metabolites, and secondary bile salts either travel to specific sites in the brain or induce responses via vagal and spinal neuronal end signaling. These microbe-derived signaling molecules also produce neuroactive molecules, such as γ-aminobutyric acid (GABA) and 5-hydroxytryptamine (serotonin or 5-HT), which further strengthen their contribution to neurological disorders mentioned earlier [1].

A vast and diverse population of microbes in the gut is associated with good human brain health. The imbalance in the gut microbiota associated with unhealthy outcomes is known as dysbiosis [8]. Gut microbes also control hunger centers and regulate appetite using orexigenic and anorexigenic hormones that stimulate satiety [9]. Dysbiosis leads to chronic neuroinflammatory conditions, forming the pathogenesis of neurodegenerative diseases (NDDs), such as PD, ALS, and AD. Metabolites of healthy microbial growth include choline derivatives, indoles, vitamins, and polyamines. They protect against colorectal cancer and irritable bowel syndrome by interfering with receptor signaling and inhibiting the release of proinflammatory cytokines or other metabolic functions, such as lipogenesis and gluconeogenesis. In contrast, pathogenic microbial metabolites contribute to disease pathogenesis and promote their progression [10]. However, at the nascent stage, research clearly stated that a healthy gut is very important for the well-being of life. According to the National Institute of Environmental Health Sciences, 6.2 million and 1 million people live with AD and PD, respectively, in the USA [11]. These numbers create a huge burden on society, seeking solutions to study the disease and its treatment options. The possibility of understanding NDDs through MGBA is of particular importance because (i) the complexity of the nervous system restricts the use of traditional methods, (ii) pathogenesis and disease progression are unclear and vary

among patients, (iii) treatment options through clinical trials have not yielded positive results, and (iv) NDD incidence increases with age and greatly compromises the quality of life [7]. The gut microbiota regulates the cellular integrity of the nervous system by facilitating the development of astrocytes and microglia. These cells are integral to neural development and neurotransmission. They also modulate neuroinflammation during brain injury and the etiopathogenesis of diseases such as ASD, AD, and PD [12]. Another important correlation between the gut microbiome and NDDs is the common factors affecting them, such as diet, sedentary lifestyle, sleep–wake cycle, stress levels, and constant noise exposure. Mouse models have shown that a normal gut microbiota reduces anxiety and increases motor activity, while specific pathogen-free (SPF) mice show anxiety-like behavior with altered expression of genes coding for secondary messengers and synaptic potentiation in the brain [13]. Lipopolysaccharide (LPS) is known to cause inflammation. Microbial LPS-induced immune memory has been observed; however, the mechanism of specific immune memory in microglial cells is unclear. The long-term effects on microglial memory remain to be elucidated [14]. The BBB is composed of pericytes (PCs), a basement membrane (BM), brain microvascular endothelial cells (BMECs), and the perivascular foot of astrocytes. The BBB plays a crucial role in regulating the entry of substances necessary for maintaining brain health and nutrients from blood sources. In addition, it limits the entry of potentially hazardous molecules and cells into the brain [15]. Further mouse model studies have found that occludin and claudin-5 (proteins produced in BBB tight junctions) expression decreased in germ-free mice and that exposure to pathogen-free gut microbiota increased, thus decreasing the BBB permeability [16]. Thus, BBB integrity is affected by the microbiome. Studies have also stated that BBB–microbiota communication starts intrauterine and progresses into adulthood.

Rodent models have been widely used to understand systemic diseases and evaluate primary drugs. The scenario of NDDs is slightly different, as they have a cognitive element in addition to molecular markers of disease progression, which cannot be compared robustly to rodent models. This limitation in animal models is reflected in the poor success rates of AD drug discovery [17]. With respect to AD, it is known that rodent models do not naturally produce amyloid β (Aβ) protein and are resistant to the pathology, thus downplaying clinical symptoms and neuronal loss [18]. Transgenic mice have been developed that overexpress the amyloid precursor protein (APP), alone or with presenilin 1 (PS1) and 2, which promote secondary Aβ plaque formation [19]. There are differences in cerebral folding, such as in the lissencephalic brain in rodents, as opposed to gyrencephalic brains in humans. Hargis and Blalock recently showed that major transgenic models are not concordant with each other and human AD. Human AD cases are not similar to those of spontaneous AD in mice and show different findings [20]. As discussed earlier, two major factors affecting gut microbes are diet and the environment. Therefore, it is questionable whether laboratory rodent diets and exposure to antigens differ from those of free-living mice or humans [21, 22]. Some studies suggested pet models, mainly canine models, because the environment, such as air and water, is the same. Starch-rich diets have also been adopted by dogs and can be used in predictive models [23]. However, other factors, such as brain versus body size, must be considered. In addition, metabolism in different species is governed by different cytochromes P450 (CYPs). This creates disparities in pharmacological and toxicological drug studies, magnifying clinical trial failures [24]. Intestinal products, such as host and/or microbial metabolites and microbial-associated molecular patterns, are transported to the liver through the portal vein and influence liver function. Simultaneously, the liver transports bile salts and antimicrobial molecules to the intestinal lumen through the biliary tract to maintain gut eubiosis by regulating unrestricted bacterial overgrowth [25]. Other models, such as *Caenorhabditis elegans*, *Drosophila melanogaster*, and zebrafish, have been established but have high translation failure rates. Establishing a gut microbiome similar to that of humans is difficult because their gut epithelial composition varies greatly, even among the same species [26, 27]. Animal models, although largely used in primitive studies, cannot be used as a solid basis to extend to human neurology. This is mainly because the cognitive and behavioral functions of *Homo sapiens* are far more complex than those of rodents and other mammals. Other factors, such as reproducibility, cost, space, time, and ethical constraints, have been recognized in animal models [28].

Reliable and robust in vitro models can be constructed to overcome problems associated with in vivo models. It may not allow surpassing in vivo models but can help increase the success rates and reliability of animal models. Three-dimensional in vitro models allow for continuous measurement/monitoring at the convenience of the laboratory for longer periods with great reproducibility and have benefits over the conventional two-dimensional (2D) models [29]. With new cell culture techniques and biomaterials, recreating in vivo conditions has become easier. Cell lines (such as Caco-2) of colorectal adenocarcinoma origin are widely used in absorption studies and transportation cross barriers. Similarly, HT-29-MTX-E12 (MTX: methotrexate) cells produce interleukins (ILs), tumor necrosis factor-α, mucus, and goblet cells. This makes it a good candidate to understand the intestinal barrier. Another cell line (T84) originating from a colon carcinoma lung metastasis exhibits brush border cell-like features and forms tight junctions through the expression of proteins such as claudins and occludin, whereas Caco-2 and HT-29-MTX-E12 are primary colon tumor cells [30].

This also equips them with higher resistance and provides excellent models to study stress on the epithelial cell lining [31]. The developed human gut models may include multiple strains of the core microbiome, as reported by El Houari et al. [32]. They successfully studied the effects of drugs, other antibiotics, and metabolic patterns in 39 combinations and individual strains [32]. Advances in laboratory techniques, such as Transwell culture plates, have allowed the cocultivation of multiple microbiome strains and intestinal lining cells. The compartments are separated by a semipermeable membrane that allows the microbiome to interact with the host's intestinal cell lining. Such studies have been used to study drug action and probiotics and establish communication between metabolites from the microbiome to the intestine and the brain [31, 33]. Hence, Transwell allows multiple cells to remain in their specific environment and still interact with other cells via chemicals, such as growth factors, which can be transported across the membrane. However, this approach presents some challenges. The intestinal lining is not only epithelial but also consists of Paneth cells, dendritic cells (DCs), and goblet cells. The brain is composed of a different environment with cerebrospinal fluid (CSF) and is covered by a tight and extremely selective BBB. Incorporation of all these cells in their correct orientation and abundance is a great task to achieve robustly in Transwell systems. On the positive side, it provides options to individualize models for patients or diseased conditions and study MGBA in pathological conditions.

This review discusses the research related to the hypothesis linking the host–microbiome to the brain and how it can affect the precipitation of NDDs. This review focuses on the following aspects.

I. The communication pathways that enable crosstalk between the gut, its microbiota, and the brain, which individually and together delve from nervous and immune systems, together with the metabolism of the microflora.
II. This review attempts to understand how these pathways affect the occurrence of NDDs, such as AD, PD, and ASD.
III. This review identifies, lists, and critically evaluates in vitro microphysiological models that have enabled the study of MGBA. This review discusses different methods to develop variable microphysiological models (spheroids, organoids, BBB, and gut epithelial barrier).
IV. This review tries to shed light on how these individual systems can come together with the help of microfluidics and Transwell systems to form a multiorgan system that will help understand disease progression and further use it to develop individualized disease therapeutic models for patient care.

Communication pathways in MGBA

The axis formed between the gut microbiota and the brain is a to-and-from communication channel. An ideal interaction between the enteric nervous system (ENS) (gastrointestinal (GI)) and the central nervous system (CNS) is essential to flourish the microbial population, and a perfectly balanced microbiome is essential for the sound functioning of the brain [34]. Multiple molecules, cells, tissues, and organs are involved in access, involving two barriers: the intestine and BBB. Numerous channels in both the gut and brain can potentially range from adaptive neuronal pathways to small molecules that are difficult to quantify.

Neural system

The nervous system comprises the CNS and ENS, which act via the autonomic nervous system (ANS) and peripheral nervous system (PNS). The ANS works autonomously without conscious effort and involves relaying signals between the central and peripheral nerves that can be further sympathetic or parasympathetic. They maintain cell, tissue, and organ homeostasis throughout the body and counter motor, endocrine, and behavioral signals [35]. ANS signaling, along with the endocrine system and the CNS, facilitates top-down communication, i.e., from the brain to gut [36]. Regular functioning and physiology of the GI tract, such as gut motility, mucus and bicarbonate production release, permeability, mucosal immune barrier, and intestinal fluid maintenance, are controlled by the ANS [37]. Pain and stress can alter the aforementioned conditions and are relayed by the ANS [38]. In a study to understand the effect of probiotics (a combination of live beneficial bacteria and/or yeasts that naturally live in the body), such as *Lactobacillus reuteri* (LR), on pain perception, the presence of LR in the diet increased the excitability and generation of action potentials (APs) in enteric sensory neurons, probably via calcium-dependent potassium ion channels [39]. Similar electrophysiological evidences, such as AP shapes, firing thresholds, the number of APs fired at twice the threshold, and passive membrane characteristics of neurons in the myenteric plexus of the ENS, were presented when germ-free mice were colonized by the gut microbiota [40, 41].

The VN is a major nerve of the ANS that connects the gut to the brain. It interacts parasympathetically and bidirectionally. Hence, any disorder in the nerve can cause CNS disarray such as NDDs or GI diseases such as irritable bowel syndrome (IBS). Afferent VNs are distributed in digestive walls without entering the epithelial layers or contacting the intestinal lumen directly. As a result, they receive indirect signals from the microbiota via metabolites or endocrine cells [34]. Bravo et al. showed that mice chronically treated with *Lactobacillus rhamnosus* (*JB-1*) had

reduced levels of stress-induced corticosterone elevation, suggesting that the microbiome, gut, and brain are connected [42]. Furthermore, these changes were not observed in vagotomized mice, clearly indicating the role of the VN in MGBA. The study also revealed an increase in $GABA_{B1}$ receptor expression, a decrease in which was observed in animal models of depression [42]. Perez-Burgos et al. supported these claims, as they found that more than 50% of afferent VNs act as interneurons for *JB-1*, receiving signals from sensory synapses [43]. Synaptic blocking by calcium channels or nicotinic blockers reduces neuronal firing [43]. Similarly, the antianxiety effect of *Bifidobacterium longum* NCC3001 (*B. longum* NC3001) was demonstrated in nonvagotomized mouse models, and *B. longum* NC3001 was able to normalize the behavior but did not modulate myeloperoxidase (a heme-containing peroxidase highly expressed in multiple inflammatory cells, including neutrophils, activated microglia, monocytes/macrophages, astrocytes, and neurons) activity or histology studies [44, 45], suggesting that vagal integrity is crucial for behavioral changes. However, the molecular pathway needs to be deduced.

Enteric neurons are indirectly activated by enteroendocrine signaling by colonic L cells called enterochromaffin cells [46]. These cells produce 5-HT in the GI tract and store 95% of it. Trp is a precursor to 5-HT. Trp unavailability in the host's body necessitates its dietary consumption. The gut microbiota ensures that Trp is available peripherally, which is required for 5-HT [1]. 5-HT also plays an important role in GI motility and secretion.

Most neuronal signaling occurs in conjunction with other neurons, inflammatory molecules, dietary compounds, metabolites, and immune and hormonal factors [47]. There is evidence of direct neuronal signaling by microbial antigens, but the degree of involvement cannot be quantified. A study evaluating the presence and response of Toll-like receptors (TLRs) in the neural plexus of murine and human intestines to viral RNA and LPS from Gram-negative bacteria revealed that TLR-3 and TLR-7 can recognize viral RNA and TLR-4 can recognize LPS. These were concentrated in myenteric and submucosal plexuses, demonstrating that antigens from the gut can directly activate the neural plexuses [48, 49]. Similarly, *JB-1* and *Bacteroides fragilis* were added to the epithelium, and the voltage was recorded. Sensory responses were recorded for 8 s and lasted for 15 min. When applied alone, the capsular polysaccharide A of *Bacteroides fragilis* also showed similar results. Neuronal stimulation by complex polysaccharides has been reported for the first time [50]. Direct neuronal signaling opens a new array for understanding MGBA.

Neurotransmitters

Microbial endocrinology has advanced in recent years, as it helps connect the MGBA. Microbes produce a range of neurochemicals typically used in human and mammalian signaling pathways [51]. Hence, it is imperative that neurotransmitters produced by gut microbes affect the host by acting on neurons or the endocrine system. Microbial endocrinology was developed as a field of research in 1993 [52]. Since then, many researchers have shown that the gut microbiota synthesizes and responds to neurotransmitters, such as 5-HT, GABA, catecholamines, histamine, steroids, and neuropeptides [53]. They are involved in brain functions such as mood regulation and cognition.

Serotonin

5-HT is produced by microbes, such as *Lactobacillus plantarum*, *Streptococcus thermophilus*, *Klebsiella pneumoniae*, and *Escherichia coli* (*E. coli*) [54]. As mentioned, about 90% of 5-HT is produced and stored in intestinal cells. A recent study suggested that for spore-forming bacterial species such as *Clostridium* spp., specific metabolites increase 5-HT levels in ECCs via 7α-dehydroxylation (important for converting cholate to deoxycholate) in the intestine, further increasing GI motility [55]. Deoxycholate is a secondary bile acid produced by the microbial biotransformation of cholate (primary bile acid), which reduces murine spontaneous contractility of colonic longitudinal muscle via a mechanism involving the expression of Takeda G protein-coupled receptor 5 (TGR5) on ECCs [56]. In another study by Hata et al., germ-free mice had low 5-HT levels in the cecum and colon, whereas other rodent models had higher 5-HT levels in the intestinal lumen within 3 d of exposure [57]. Germ-free mice have 50% conjugated (inactive) 5-HT, whereas conventionalized mice have major unconjugated, active 5-HT. The observed deconjugation showed that the gut microbiome plays an important role in activating neurotransmitters [57]. It is also known that microbes can produce 5-HT, and the gut microbiome is modulated by SCFAs or secondary bile salts, which act on the host's Trp hydroxylase (TPH), an enzyme necessary for 5-HT production. TPH exists as two different isoforms, TPH1 and TPH2, responsible for peripheral and central 5-HT, respectively [53, 58]. Another pathway, such as the kynurenine pathway (KP), is essential for producing cellular energy in the form of nicotinamide adenine dinucleotide (NAD^+). As energy requirements increase significantly during an immunological response, KP is an important immune system regulator. KP is thought to play a role in the internalization of Trp into the intestine [59, 60].

GABA

GABA is a major inhibitory neurotransmitter formed by converting the amino acid glutamate into GABA. This conversion occurs in humans, hosts, and microbes. Extensive literature supports GABA involvement in the CNS and sleep disorders [53]. Fermented strawberry juice contains millimolar levels of GABA (262 mmol/L) [61]. Although the exact mechanism of action of GABA is unknown, germ-free mice have significantly lower GABA levels in their intestinal lumens [62]. *JB-1* (a widely used probiotic strain) reduced anxiety and depression-like disorders in mice and gave significant weight to VN involvement, as vagotomized mice were unaffected [42]. Hence, GABA may act specifically via the VN in *Lactobacillus* species. Another study on a lipopolypeptide analgesic synthesized by *E. coli* strain Nissle 1917 (EcN) reported that the strain produced C12AsnGABA*OH* (analgesic polypeptide), which can cross the epithelial barrier. It inhibits the calcium flux propagated by nociceptor activation via the $GABA_B$ receptor. This blocks visceral hypersensitivity, which sends a pain signal [63]. A similar study of abdominal visceral pain was conducted by Pokusaeva et al. [64]. Oral administration of *Bifidobacterium dentium* (which harbors the *gadB* gene) produces GABA via glutamate decarboxylation. It modulated the sensory activity of neurons in a rat model of visceral hypersensitivity [64]. Targeting GABAergic microbes may be a new approach to treat abdominal pain. Other methods, such as ketogenic diets, increase GABA levels in the CSF of children with refractory epilepsy [65].

Dopamine and catecholamines

Dopamine is an important neurotransmitter and precursor of other catecholamines such as epinephrine and norepinephrine (NE). It is well established that catecholamines are involved in fight-or-flight mechanisms, alertness, motivational rewarding behavior, and decision-making [66]. In the early 2000s, pathogenic *E. coli* had an increased growth rate in the presence of dopamine and NE [67]. Others, such as *K. pneumoniae*, *Pseudomonas aeruginosa*, and *Shigella sonnei*, also showed improved growth rates in the presence of NE in vitro [68]. Researchers have shown that NE production renders the host susceptible to bacterial virulence genes, thereby inviting infection. NE signals to bacteria have chemotactic effects that drive the migration of bacteria toward the host intestinal mucosa [69]. Catecholamines also contain siderophores that release iron via protein sequestration, making them a hotspot for bacterial growth [70]. Through its interaction with the bacterial quorum-sensing histidine kinase, NE can operate as a quorum-sensing molecule to promote the expression of bacterial virulence genes [71]. Although their involvement has not yet been confirmed, evidence suggests that they play a role in catabolism. A recent in vivo study by Asano et al. demonstrated that germ-free animals have low NE levels in the fecal lumen, which can be restored by colonization with a microbiota mixture of 46 *Clostridium* species, suggesting that microbiota levels also influence NE levels, unlike previous studies that showed that NE levels affect microbial infestations [72]. Understanding these communication pathways and their links to disease phenotypes may allow the development of microbiome-mediated therapies to control these targets and potentially cure diseases with significant unmet needs, such as those affecting the ENS/CNS.

Immune system

The immune system and microbiota work together to develop the immune system and the host–microbiome [73]. Immunity mediators include chemokines, ILs, cytokines, and other molecules released in response to microbial antigens/epitopes. Many immune modulators play an important role in neuroinflammation, as observed in many NDDs and neurodevelopmental disorders [35]. Recently, germ-free mice displayed defects in microglia with altered phenotypes and cell incidence, leading to impaired innate immune response. If the microbial population diversity was reduced, defects in microglial structures could still be observed. During recolonization, partial reconstruction of microglial features was performed. Erny et al. determined that SCFA and other bacterial fermentation products controlled microglial well-being [74]. These SCFAs also increase Aβ proteins in AD in in vivo models [75]. SCFAs could potentially be promising targets for treating neurological disorders from an inflammatory point of view. Furthermore, an extensive review by El Aidy et al. showed that increasing animal model evidence has been generated, suggesting that the immune system and endocrine signaling play a major role in MGBA crosstalk [76]. Innate immunity is the first line of rapid and nonspecific defense against pathogens that the body deploys within hours after encountering antigens. It lacks immunological memory and cannot recognize the same pathogen upon exposure. In contrast, adaptive immunity is antigen-specific and involves a delayed response after antigen exposure. It is characterized by immunological memory that allows the host to mount a faster and more efficient immune response upon subsequent encounters with the same antigen [77]. Adaptive immunity comprises T and B cells specializing in identifying, fighting, and memorizing antigens. These cells also play important roles in allergies, asthma, and the immune hypothesis of schizophrenia/depression [35]. As discussed earlier, the immune system can detect LPS via TLRs and reach the brain via circulation [48]. This process can initiate a cascade of neuroinflammatory reactions.

The effects of infectious microbes on behavioral patterns are well documented. For example, administering infectious microorganisms, such as *Campylobacter jejuni*, to mice induced anxiety-like behavior [41]. Similarly, peripheral administration of cytokines (proinflammatory) recorded disturbed rodent behavior, similar to sickness, including fatigue, reduced appetite, and sleep disturbances [78]. In healthy humans, microbiota homeostasis helps to maintain an active immune system. Similarly, microbiota can protect the host from *E. coli* infection sepsis. Antibiotic treatment results in dysbiosis, reduced IL-17 levels, and granulocyte-colony-stimulating factor production [79]. Some microbes also positively regulate the release of chemokines, such as the oral consumption of *Bifidobacterium infantis* in humans and increased IL-10 expression in human peripheral blood [80]. Another study showed that germ-free mice could not produce type I or II interferons (IFNs). This compromises host immunity against viruses [81].

Thus, it is clear that the immune system and the gut microbiota are synchronized. Insufficient microbiota makes hosts susceptible, whereas their presence increases the ability to ward off infections by increasing ILs and IFNs. Therefore, it is likely that the intestinal microbiome closely regulates inflammatory responses in the host and disruptions to this microbial balance, particularly in childhood, because early life stress can induce multiple changes across the brain–gut axis, resulting in a chronic inflammatory state that can lead to adverse changes in mood and behavior [82, 83].

Microbial metabolism

Microbial metabolites act as chemical signals to human cells, affecting cellular functions and providing cues for microbial growth and cellular functions [55, 74]. SCFAs and Trp metabolism are among the most crucial pathways.

SCFAs, such as butyrate, propionate, and acetate, are primary metabolites produced by the bacterial fermentation of dietary fibers and resistant starch. Locally, they communicate with epithelial cells and the ENS, as both are abundant in the gut [84]. SCFAs activate or bind to free fatty acid receptors 2 and 3. These receptors control peptide YY and glucagon-like peptides, which are anorectic hormones. They are involved in satiety, thus forming a link between SCFAs and food intake [85]. A possible distal way to communicate with the brain is through the BBB. SCFAs (butyrate and propionate) reach the bloodstream with the help of monocarboxylate transporters via passive diffusion. The same was observed for the BBB. It may cross the BBB, access the CNS, and be taken up by transporters in glial cells and neurons [86]. SCFAs also induce epigenetic modifications and regulate inflammation [87]. Butyrate and, to a lesser degree, propionate have inhibitory activities on histone deacetylase (HDAC). This inhibition leads to an increase in histone acetylation and active neuronal transcription. This change in genetic regulation improves the memory and regeneration of neurons in animal models of learning [88, 89]. Behavioral studies in rats and mice have also revealed that SCFA administration induces different effects. For example, MacFabe et al. concluded that intraventricular administration of propionic acid induced autism-like behavior, although the underlying mechanism could not be deduced [90, 91]. Another study showed antidepressant-like behavior upon the systemic injection of sodium butyrate. It changes the expression of brain-derived neurotrophic factor (BDNF) and acetylated frontal cortical and hippocampal histones [92].

The presence of SCFAs seems to be subtle, but SCFAs generated by most gut microbiomes, such as *Akkermansia muciniphila*, *Faecalibacterium prausnitzii* (*F. prausnitzii*), *Eubacterium hallii*, *Bacteroides* spp., *Bifidobacterium* spp., and *Clostridium* spp., result in epigenetically regulated gene expression because of HDAC inhibition by SCFAs [93, 94]. Given the epigenetic involvement of SCFAs, they can be viewed as an entity, as they exhibit effects similar to those of the host, such as acetylation and histone modification [41] (Fig. 1).

MGBA involvement in neurological diseases

As the credibility of the relationship between the gut microbiome and the brain via MGBA has been established over years of research, it has been suggested that MGBA plays a significant role in neurological diseases and their occurrence and progression. Many neurological diseases have other factors such as stress response, environmental factors, genetics, and coping mechanisms. These factors also affect gut health. Hence, the correlation will allow a better understanding of the disorders and open gates to new therapeutic targets for the disease. There are various neurological disorders such as depression, anxiety, AD, PD, multiple sclerosis (MS), ASD, stroke, brain injury, and attention-deficit hyperactivity disorder (ADHD), to name the top layer of the list. This section discusses MGBA involvement in three main neurological disorders: a neurodevelopmental disorder called ASD and two NDDs, namely AD and PD.

ASD

Autism is a neurodevelopmental disorder characterized by core symptoms such as lack of sociability, repetitive behavior, and difficulty in communicating. GI symptoms also contribute to the morbidity. The severity of GI symptoms is highly correlated with that of ASD symptoms. Patients with ASD often fall prey to gut dysbiosis, leading to bloating, diarrhea, and constipation; however, there is limited robust evidence to support this theory and conclude the results [1].

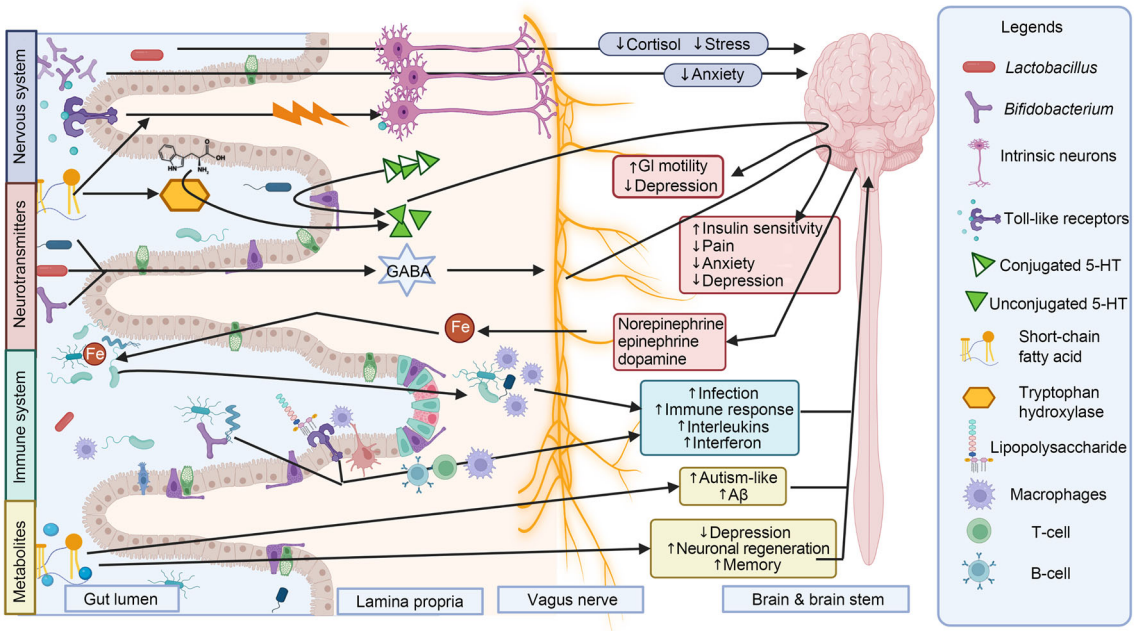


Fig. 1 System and pathways involved in MGBA. MGBA is governed by different systems and pathways. The nervous system includes enteric nerves, VNs, and neurotransmitters released from brain and gut synapses. The presence of microbiota in the gut attracts the immune cells to sequester LPS and cell debris that can further signal the nervous system. Microbial metabolic products and peptides also contribute directly to hormonal and neuronal pathways and secretions. Chemical and neural signals reach the brain and in turn affect the gut health such as motility and mucus secretion. MGBA: microbiota–gut–brain axis; VNs: vagus nerves; LPS: lipopolysaccharide; GABA: γ-aminobutyric acid; GI: gastrointestinal; Aβ: amyloid β; 5-HT: 5-hydroxytryptamine

An open-label study of 18 children treated with the oral nonabsorbable antibiotic vancomycin showed a significant (80%) reduction in GI symptoms and behavioral symptom improvement. The improvement persisted for eight weeks after treatment [95]. Although antibiotic treatment cannot be a permanent solution, the improvement suggests that there is some GI microbe eradication that helps alleviate the symptoms. Beneficial bacteria, such as *Bifidobacterium*, were reduced in abundance, whereas pathogenic strains, such as *Desulfovibrio* and *Clostridia*, were more abundant. Góra et al. determined that *Clostridium perfringens* isolated from the fecal matter of children with ASD had a higher expression of *cpb2*, a gene that encodes clostridial toxin B [96]. Toxin β2 has been associated with GI disorders such as diarrhea, and its high levels in individuals with ASD may provide insights into comorbidity [95].

Preclinical studies have also provided insights into the relationship between MGBA and autism. Shank3 and Black and Tan Brachyury (BTBR) mouse models are predominantly used in ASD studies. In a preclinical study using Shank3 transgenic mice, *Lactobacillus*, *Prevotella*, and *Veillonella* were notably reduced. Treatment with *L. reuteri* improved the social behavior of male mice, which also showed increased oxytocin expression. There were no changes in oxytocin levels or behavioral patterns in female mice. Reversal by *L. reuteri* was possible via the VN [97, 98]. BTBR mice also displayed population loss of *Bifidobacterium* and *Blautia*. They were also deficient in bile moieties, probably because of their altered metabolism. In addition, they exhibited prolonged intestinal motility, which is consistent with constipation. Studies have revealed changes in intestinal wall permeability [99]. Individuals with ASD have higher LPS levels and thus higher levels of IL-6, a neuromodulating cytokine [100]. A study of individuals with ASD and their siblings on intestinal permeability and pyrosequencing of the microbiome indicated that intestinal permeability increased in ASD and non-ASD individuals, suggesting that this is a course of pathogenesis rather than a consequence of autistic behavior. The microbial population showed a higher level of *Bacteroidetes* in individuals with ASD and predominant *Firmicutes* in the control group [101]. However, the specific set of microbes that cause autism remains unclear.

There are other factors to consider. ASD children have higher antibiotic usage, diet restrictions, breastfeeding, and environmental comfort than non-ASD children, which can very well question the findings [102]. In summary, these studies provide promising evidence indicating a more direct role of the MGBA in ASD pathogenesis than previously considered. This area of research has received greater attention in autism in recent years and will generate more interest and fruitful results in the coming years, which may impact treatment strategies for ASD.

PD

PD is a progressive neurodegenerative disorder. Symptoms include movement rigidity, tremors in the extremities, and distinctive gait due to motor impairment [35]. This occurs due to the death of dopamine-generating cells in the substantia nigra, thus reducing dopamine levels in the brain [41]. Aggregation of α-synuclein is also observed in mucosal and submucosal nerve fibers and ganglia [6]. This finding suggests a connection between the gut and PD progression, primarily via the VN. By the time physical and motor impairments are noticeable, pathology has advanced. The severity and frequency of constipation increase the risk of developing PD. IBS-like symptoms and PD are comorbid [103]. Constipation is the earliest sign of developing almost 15 years before the onset of motor impairment [1]. Evidence suggests that the population and diversity of the microbiome are associated with MGBA [104]. Appendectomy has recently been considered a prophylactic treatment for PD initiation [105].

Preclinical studies have helped to draw a subtle line of crosstalk between PD and gut health. Fecal microbiota transplant (FMT) from a PD patient into germ-free mice resulted in mice showing two main symptoms: motor deficits and neuroinflammation [106]. Bhattacharyya et al. studied the effects of altered intestinal permeability [107]. They reported that LPS released by gut bacteria traveled and modulated α-synuclein by forming nucleating intermediates. Nuclear magnetic resonance studies have revealed the formation of an LPS-binding motif that affects cellular internalization and cytotoxicity, suggesting an alternative pathway that exasperates PD [107]. α-Synuclein transport from the gut to the brain is assisted by the microtubular action. The same study also demonstrated that α-synuclein from a brain lysate enters the dorsal motor nucleus of the brainstem via the VN [108]. Another important study showed high levels of proinflammatory bacteria *Bacteroidetes*, *Proteobacteria*, and *Verrucomicrobia* in the feces of PD patients [104]. Similarly, the relative population of *Enterobacteriaceae* was positively related to gait difficulty and unstable posture [109]. Although there are many studies, PD patients also have regular medication, which can chronically affect the microbiome. A large cohort study of 197 patients with PD and 130 healthy individuals suggested that PD medication altered the microbial population, including *Bifidobacteriaceae* and *Christenesenellaceae*. Predictive functional probes suggest many alternative pathways, owing to xenobiotic metabolism [110].

Extensive studies have been performed in animal models that clearly state a bilateral relationship between the gut and PD progression. However, the set of microbes, targeted pathways, and proteins was different in each study. Some studies have suggested that treatment with fermented drinks acts as a probiotic and can help relieve constipation among PD patients [35]. Much research must be undertaken to understand the specific effects of microbial species on motor and nonmotor symptoms.

AD

AD is a leading cause of dementia in the elderly population. The disease is characterized by the accumulation of Aβ plaques and hyperphosphorylated tau proteins in the cortex, followed by its progression to the hippocampus and other brain regions [35]. As the disease advances, there is visible memory loss, confusion, inability to behave in a manner, disorientation, and lack of motivation and self-care [41]. Strong evidence exists that atypical inflammatory signals degrade neurons and allow the agglomeration of amyloid plaques, thus initiating the disease cascade [111]. The presence of inflammatory signals also suggests the possibility of a pathogenic/microbial origin of AD, and evidence has been collected over the years [35]. Reports have also suggested that amyloid plaques are of microbial origin [112]. A possible relationship between MGBA and amyloid plaques has been mapped by Cattaneo et al. [113]. Their studies showed an increase in proinflammatory cytokines, such as IL-6 and IL-1β, among others, which was positively correlated with an increase in the abundance of MGBA taxons, such as *Escherichia/Shigella*, in cognitively impaired patients. A reduction in the anti-inflammatory activity of *E. rectale* was also observed. Peripheral inflammation has been observed in cognitively impaired and amyloidotic patients [113]. Similar peripheral systemic inflammation was corroborated by Aso et al. [114]. When AD was induced in mice, inflammatory responses were observed in the brain and blood, confirming the above case [114]. Similar to PD patients, an analysis of 25 AD patients with mild dementia showed reduced *Firmicutes* and *Bifidobacterium* and increased *Bacteroidetes* in the gut [115].

Animal model studies of germ-free APP-PS1 mice have low Aβ pathology compared to conventional models with the same background, indicating that gut microbiota plays a role in Aβ formation and AD pathogenesis [101, 102]. Administering *Bifidobacterium breve* strain in the Aβ intracerebroventricular model prevented cognitive dysfunction, restored partial memory, and reduced inflammation [116, 117]. Another probiotic cocktail of *Lactobacillus acidophilus*, *Lactobacillus fermentum*, *Bifidobacterium lactis*, and *B. longum* resulted in learning and memory problems in an intrahippocampal Aβ model of AD [118].

This study lays the foundation to point out that there is a significant relevance in learning MGBA with respect to AD progression. Better understanding will allow for new cures or adjunct therapies to help maintain a good quality of life (Fig. 2).

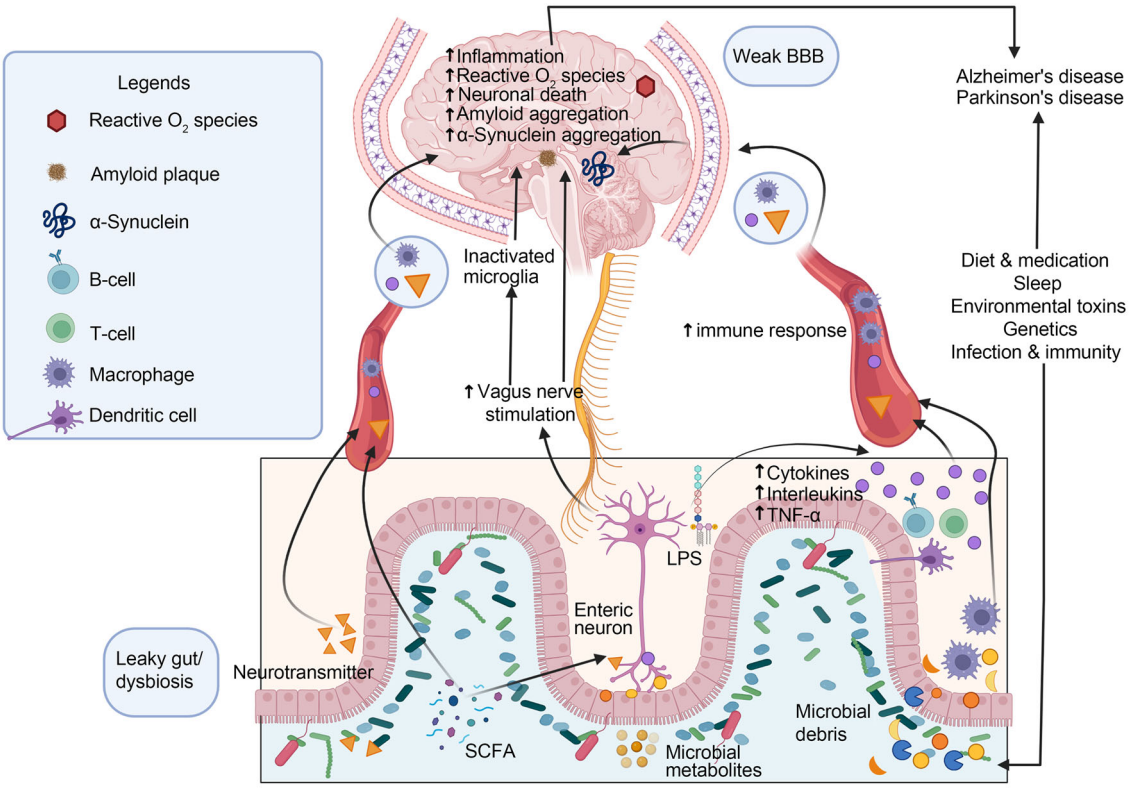


Fig. 2 MGBA in NDDs. Dysbiosis in the gut microbiome affects the nervous and immune systems, which alter brain structure and functioning via inflammation and stress signals. A leaky gut often results in a weak BBB, which leads to protein aggregation and causes NDDs. MGBA: microbiota–gut–brain axis; NDDs: neurodegenerative diseases; BBB: blood–brain barrier; TNF-α: tumor necrosis factor-α; SCFA: short-chain fatty acid; LPS: lipopolysaccharide

In vitro remodeling of the human MGBA

The human physiology and pathophysiology of various diseases connecting the brain and gut are discussed here. Under diseased conditions, the interconnected axis pathway is awry. As discussed earlier, animal models do not sufficiently represent the complexity associated with human neurological conditions, especially translation from one species to another. There is a growing awareness among scientists to use fewer animal models and move toward more adaptive in vitro models. In vitro models have the innate ability to be performed in the laboratory and are standardized, controlled, and inexpensive. Remodeling MGBA in vitro is a humongous task, as multiple types of cells are involved and the microenvironment of each is different.

A miniaturizing or microphysiological system is an important concept, as it can provide correct results in fewer reagents, less space, and fewer resources. Minimizing the host–microbiome using advanced cell culture techniques, such as Transwell systems, allows the growth of adhesion-dependent cells and coculture with other cells in the axis, such as the gut microbiota or neurons [119]. Progressing from 2D monolayer cultures to three-dimensional (3D) spheroids and organoids to using the Transwell system and the latest organ-on-a-chip, in vitro disease modeling has come a long way. The introduction of 3D constructs using hydrogels allows the construction of scaffolds that mimic in vivo spatial features and induce physiological actions from cells, such as nutrient uptake and shear stress that stimulates cell division and growth. Amalgamating these with advanced sensory equipment for pH, O_2 concentration, and pressure has allowed scientists to have better control over the experiments. It also allows tweaking protocols at the molecular level [120].

Before diving into a cell patterning microphysiological system, some crucial factors must be considered:

(i) selecting a microbial sample that aptly represents all;
(ii) choice of culture medium that guarantees the survival and optimal growth of cells and the microbiota;
(iii) maintaining optimum environmental conditions, such as pH, O_2 levels, peristalsis, and hormones;
(iv) surface to attach and grow—a physical substrate for microbial adhesion;
(v) shear stress studies to ensure that the system is sufficiently robust to withstand fluid retention, movement, and circulation; and

(vi) culture medium and its volume to sustain coculturing microbiota and mammalian cells (anoxic–oxic interface) [29].

Spheroids

3D spheroids are a novel investigative platform used for in vitro neuronal development, drug delivery, and the availability and pathogenesis of various diseases. Small cellular aggregates better represent cells in vivo without any adjuvant material. It allows the study of cell–cell interactions and the functionality of the microenvironment. However, they do have a drawback in that they lack vasculature. Regular blood flow provides nutrients to cells and collects metabolic waste, thereby maintaining homeostasis. An enteric neuron spheroid was constructed by Garcia-Corral [121]. H9 human embryonic stem cells (hESCs) were differentiated into the enteric neural crest (ENC) and further into enteric neuronal cells. They were cultured in an ENS basal medium and supplemented in ultra-low-attachment plates to induce spheroid formation. The neural crest was pure, and it formed clear-edged spheres and detached from the bottom to mature further. Calcium imaging and immunofluorescence were used to confirm the functionality of neural spheroids. They also observed that β-III tubulin filaments branched from the crest [121]. Another study has used postnatal rat cortical cells. Spheroids were formed using 2% agarose and poured into micromolds, resulting in U-bottomed microwells. They demonstrated that neurons and glia with laminin were formed, and the structure showed electrical conductance and synaptic activity [122]. Another study on cortical spheroid development was conducted by Boutin et al. [123]. Cortical cells were seeded at 8000 cells/microwell. Molten agarose was lined on the walls of the wells and equilibrated with cortical medium. Endothelial cells (ECs) within the cortical cells were assembled in a capillary-like network [123]. These 3D spheroids are not only used to learn neuronal functions, but they can also be applied to microfluidic or Transwell systems that allow dynamic or static studies of the disease, respectively. Park et al. developed a model to study Aβ plaque toxicity. Spheroids were formed in concave cylindrical wells and studied using microfluidic chips. Based on the neurospheroid size, their differentiation into neurons and toxicity to Aβ were studied [124]. Researchers have also used tools such as immunostaining and flow cytometry to determine the integrity and functionality of spheroids for a better understanding of drug mechanisms in 3D cultures [120]. Another way to adopt Aβ plaques in spheroids could be using viral mutation of human neural progenitor cells (hNPCs), which produce more Aβ plaques extracellularly, thus forming an aggregation microenvironment around the sphere. This method was adopted by Kim et al. for a 3D model of AD inside a well insert, available with a detailed protocol in Ref. [125]. PD has been studied using human midbrain-derived neural progenitor cells (hmNPCs). Neurospheroids were developed by growing them in an aggregation medium and then in a differentiation medium. Electrophysiological, phenotypic profiling, and molecular studies have demonstrated the presence of dopaminergic neurons and astrocytes [126]. Spheroids are useful in mimicking and studying the BBB, as they can form tight junctions. For example, Cho et al. evaluated cell-penetrating peptides (CPPs) by making astrocyte core spheroids lined with PCs and ECs. These models may be applicable to estimate the bioavailability of therapeutics for NDDs. The pathological effects of NDDs on the structural integrity and function of the BBB can also be studied by carefully curating the proteins and extracellular matrix (ECM) around the spheroids [127].

Neural spheroid production is also laborious. As shown by Garcia-Corral, protocols require several optimization steps to yield robust results and minimize contamination [121]. Furthermore, he noted a low induction rate of differentiation of ENCs into neuronal cells, which reduces the efficiency of the process. He also suggested testing for more identification markers to pinpoint the neuronal subtype and tests. Methods or protocols to test the spontaneity and synchronization of neuronal networks have yet to be developed, validated, and standardized. Dingle et al. [122] and Boutin et al. [123] also acknowledged the difficulty in sculpting a complex multicellular neural culture. It requires expertise and resources and provides only a primary 3D model for understanding neurobiology and diseases. Urich et al. tri-cultured ECs, astrocytes, and PCs to study vasculogenesis but found that ECs surrounding the spheroids do not accurately represent in vivo vasculature [128]. The use of nonnative proteins, such as Matrigel or agarose, alters the in vivo ECM and microenvironment, potentially altering cell–cell signaling and tubule formation. The applicability of spheroids in microfluidic chips was evaluated by Park et al. [124]. Although the study was able to replicate the interstitial flow rate using osmotic pumps, the spheroid itself was only made of cortical neurons, without the influence of the ECM or other cells such as astrocytes and PCs [124].

A comprehensive literature review reported that neuronal cells and their differentiation can help build a near-to-the-real model of neuron clusters and their working. However, the gut epithelium is crypt-like, with villi with epithelial layers and mucus-producing cells. Hence, spheroids do not accurately represent the intestinal barrier. Recent advances in bioprinting have enabled mimicking tunnel-like wells that represent the epithelial lining [129]. This approach was used in a model with a cancer coli-2 cell line (Caco-2) and colon-derived ECM. The bioprinted model showed spontaneous 3D morphogenesis without the need for external cues. Cells later aggregated and differentiated into functional cells, such as

Table 1 List of spheroid models generated to study neurodegenerative diseases

	Targeted disease	Cell line/cell type	Type of spheroid	Growth conditions	Advantages	Limitations	References
1	AD	Prenatal rat cortical neurons	Monocultured spheroid with amyloid β	Ten days	Application in microfluidic chips	Use of artificial proteins disrupting signaling; Not studying multicellular signaling	[124]
2	AD	iPSCs derived from AD patients	3D cortical spheroids	Seven days in induction medium, 21 days in neural medium with growth factors, and 14 days in E8 medium with ROCK inhibitor	First iPSC-derived AD model; Proven applicability in drug screening; Patient-derived model that can track disease progression and therapy	Diffusion of the drug across the spheroid being reduced and highly sensitive to the spheroid diameter; Longer time duration to achieve uniform bioavailability in the spheroid	[132]
3	AD	Prenatal rat cortical neurons	Neurospheres using concave microwells formed by PDMS	Seven days in normal media, three days in amyloid β-containing media	Achieving 3D networking between spheroids; Ability to control the size of the spheroid	Formation of the spheroid in artificial PDMS wells possibly not an accurate representation	[133]
4	PD	Human midbrain neural progenitor cells	Monoculture	Seven days in aggregation medium, one day in differentiation medium, and 18 days in maturation medium	Efficient differentiation with functional dopaminergic and GABAergic receptors	Inability to be viable for long duration; Limited phenotype and availability	[126]
5	PD	SH-SY5Y SH-A53T	Monocultured on scaffold-free agarose micromold	Fourteen days	Information about cell lines with mutated overexpressing α-synuclein protein	Overexpressing genes in a neuroblastoma cell line not always representing the etiopathogenesis of PD	[133]

Table 1 (continued)

	Targeted disease	Cell line/cell type	Type of spheroid	Growth conditions	Advantages	Limitations	References
6	TS (characterized by ASD)	hPSCs and H9	hCSs and hSSs	hCS: five days in hPSC medium, 19 days in neural medium with GFs; hSS: 24 days with specific signaling pathway inhibitors and agonist	Forebrain assembloid developed using hCSs and hSSs; Studies on migration of interneurons and functioning microcircuits; Identifying transcriptional changes associated with TS subjects	Complicated multi-step process that is hard to standardize for individual subjects	[134]
7	ASD	iPSCs	iPSC-derived NPCs to form spheroid with *CHD8* gene knocked out using CRISPR/Cas9	Five–six days in mTeSR1 medium, 2 days in N2 medium; Formed embryoid bodies used after six days, and grown in NBF for two days; Neural rosettes pooled and aliquoted till NPCs reach 50% confluence; NPCs moved to differentiation media after two days and maintained up to eight weeks	Study of neurodevelopmental stages with high risk genes; Exposure to xenobiotics like drugs and pesticides	Limited metabolism in in vitro model needing artificial metabolites to be added in the culture	[135–137]
8	ASD	hiPSCs	hCSs	Four days in KOSR media with SMAD inhibitors and 25 days in media with GFs, grown for one day more; spheroids harvested at Day 7, 17 or 30	Imaged intact hCSs with ALSM; Study of brain development and abnormality in neural rosette from ASD patients	ALSM having limited spheroid size; Standardization of volumetric quantification being difficult but imperative to compare large data sets	[138]

AD: Alzheimer's disease; PD: Parkinson's disease; ASD: autism spectrum disorder; TS: timothy syndrome; iPSCs: induced pluripotent stem cells; hPSCs: human pluripotent stem cells; hiPSCs: human-induced pluripotent stem cells; 3D: three-dimensional; PDMS: polydimethylsiloxane; hCSs: human cortica spheroids; hSSs: human subpallium spheroids; NPCs: neural progenitor cells; *CHD8*: chromodomain helicase DNA binding protein 8; ROCK: rho-associated kinase; GFs: growth factors; mTeSR1: feeder-free maintenance medium for human embryonic stem cells and hiPSCs; NBF: neutral buffered formalin; KOSR: knockout serum replacement; SMAD: suppressor of mothers against decapentaplegic; ALSM: airy-beam light sheet microscopy

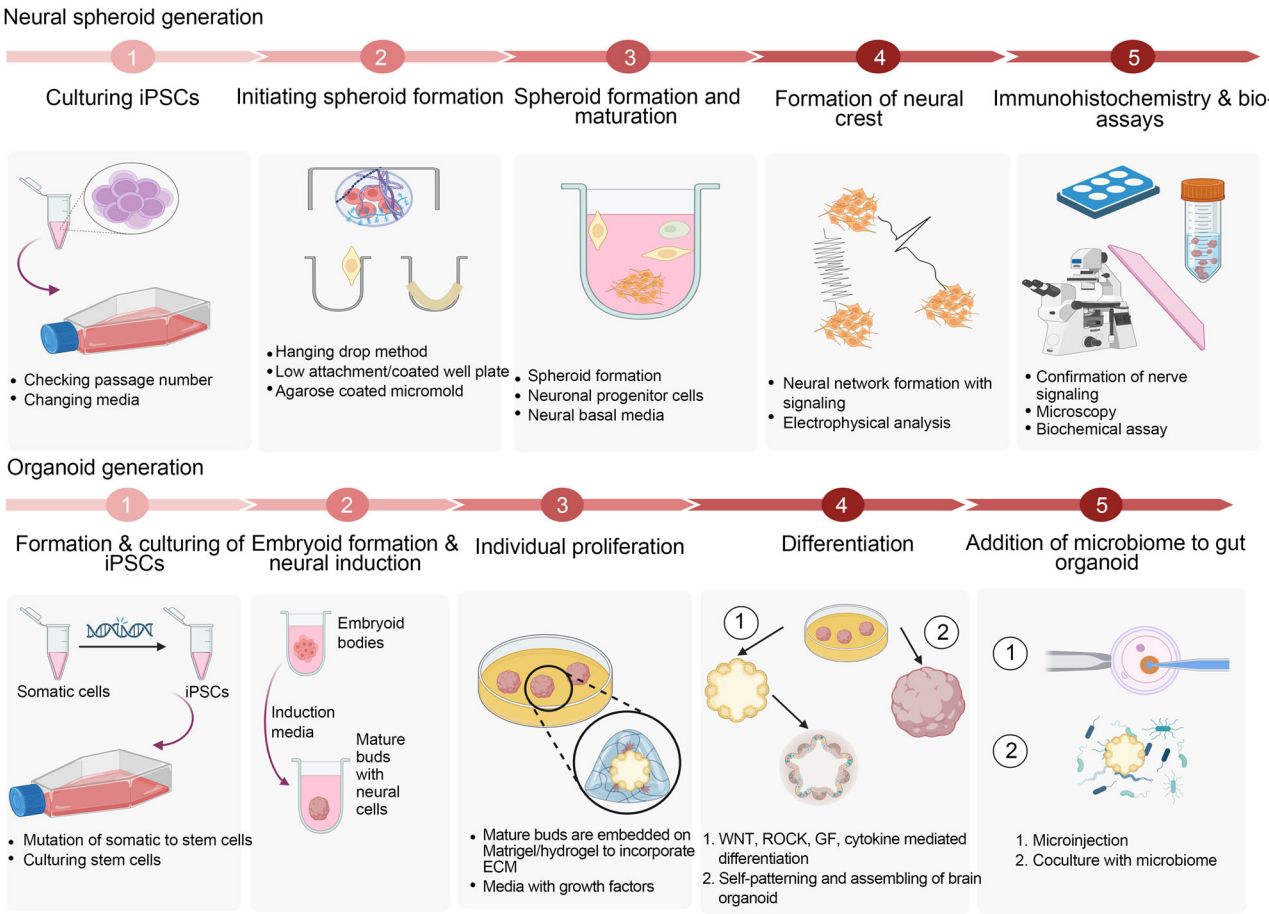


Fig. 3 Spheroid- and organoid-based neuronal models. Spheroids are generated from pluripotent cells in micromolds, ultra-low-attachment plates, or polymer-coated wells, which allow cell aggregation. A maturation medium allows the formation of neural crest cells, with neuronal signaling and receptors, which are modified depending on the required disease model. Organoids are similarly grown from buds embedded in an ECM-like material. Medium components are carefully selected and optimized for lumen formation and organoid functioning. Tools such as microinjection are used to introduce microbiota in the gut organoid. ECM: extracellular matrix; iPSCs: induced pluripotent stem cells; WNT: wingless-related integration site; ROCK: rho-associated kinase; GF: growth factor

EECs, which were identified using cellular markers such as chromogranin A [130]. Spheroids alone cannot accurately represent a multiparty system such as MGBA, but careful construction with the right cell type and ECM can build near-accurate models of brain cells. These can be further used in Transwell systems or microfluidic chips to represent the brain compartment of the model. Monocultured spheroids from different brain regions can be cultivated to form complex assemblies [131]. Although spheroids are 3D structures, real-life organ systems are not all spherical; they are multicellular constructs forming a lumen, such as the lungs, stomach, and small intestine. Lumen formation is achieved with the development of organoids (Table 1, Fig. 3).

Organoids

Different types of primary cells come together to form the intestine, including enterocytes, goblet, Paneth, and tuft cells. Forming a spheroid is impossible and unrealistic because the in vivo cellular arrangement is different. Organoids are 3D multicellular agglomerates that propagate indefinitely in specific microenvironments and growth conditions. Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) can differentiate into intestinal organoids [139, 140]. These self-assembled tissue systems recreate the architecture, cellular specificity, and function in vitro. They also can last longer than spheroids do [129].

The basic process involves embedding pluripotent stem cells (PSCs) in Matrigel as a local cell growth and differentiation niche. This was performed by Lancaster et al., in which embryoid bodies were cultured in Matrigel in a growth factor-free medium. They were then cultivated in spinner flasks. The organoids had cortical layers, as observed in the brain, and were further used in disease models such as microcephaly [141]. Another attempt was made to recreate the midbrain by Jo et al. hESC and H9 cells were seeded

in Matrigel. A rho-associated kinase (ROCK) inhibitor was added to allow uniform differentiation, and midbrain patterning factors (sonic hedgehog (SHH-C25II) and fibroblast growth factor 8 (FGF8)) were added after four days. When buds started to extrude from the gel, they were embedded in Matrigel with reduced growth factors and a medium containing SHH-C25II and FGF8. Subsequently, they were removed and planted in low-attachment plates for individual growth using differentiation media. They found that midbrain organoids have distinct, electrically active neuronal layers. They also produce dopamine and neuromelanin granules, as found in the substantia nigra [142]. The substantia nigra is also significant in NDDs, such as PD and AD. This model can be further developed to study the specific effects of coculturing gut microbiota.

A de novo generation of gastric organoids was developed by McCracken et al. They used human PSCs and manipulated genes involved in fibroblast growth factor (FGF), wingless-related integration site (WNT), and endothelial growth factor (EGF) signaling pathways to generate human gastric organoids. The organoids formed gastric glands, pit-like villi, and proliferating zones in mucus-producing cells. They also used the model to identify signaling pathways with *Helicobacter pylori* with respect to peptic ulcers and modeled its pathogenesis. The developmental stages of organoids have provided insights into the development of antrum-like structures in mice [143]. Because the gastric lumen is in the center, studies to deliver microbiota or metabolites are performed with the help of microinjection, which delivers them inside the lumen without disturbing the integrity of cell layers [144]. However, this requires a specialized skill set for embryology and specific equipment, which is troublesome. It can also compromise the organoid wall integrity.

The host–microbiome and gastric enteroids were previously studied by Noel et al. [145]. They developed intestinal enteroids and cocultured them with macrophages to understand the immune signaling and barrier function when the system integrated the pathogen *E. coli*. The epithelium and macrophages communicate via cytokine release. After infection, phagocytosis and the integrity of the epithelial barrier revealed a well-coordinated response between the host enteroid and immune cells [145]. It could be postulated that immune signaling involves the brain, which was not observed in this study. This also provides the scope to further develop the system by coculturing it with brain organoids and studying their interactions. Although there is much evidence of organoid development, little evidence or research shows the coculture of the brain and gut together to establish a robust MGBA.

Organoids are among the most promising innovations for in vitro model development. They mimic physiology, multicellularity, and microenvironment to a large extent. Genes can be manipulated using molecular tools such as CRISPR/Cas9 to drive PSC differentiation in a certain way. There is also complex crosstalk between host cells and the microbiome in MGBA. Developing hydrogels and silk scaffolds has provided a good niche and adhesive properties for cell growth [146]. The drawbacks of this system include variability and investment in developing a disease model. Their shapes and sizes may vary, making it difficult to standardize and analyze data generated across various instruments [147]. Furthermore, in order to be similar to those in vivo and be sustained, organoids need vasculature that provides oxygen and nutrients; otherwise, they develop into a static model and necrotic center. This hinders the development of an age-related functional model [148]. It is noteworthy that NDDs are often diagnosed in later stages, whereas most organoid models depict the early markers of the diseases. This is a prospect for including and constructing models to help diagnose mid- and late-stage diseases. Coculturing attempts should include immune cells resident in the ENS, such as microglia, as assembled by Ormel et al., which play an important role in NDD progression [149]. In the future, when methods are standardized, organoids can be patient-derived to understand their progression and find the most efficient therapeutic path. The disease model cannot be generalized, especially for neurological disorders, because it differs from person to person. As discussed earlier, the microbiota is specific to patients' diet, environment, and medication. Hence, it may seem like a huge investment in developing personalized disease models. However, the integration of host–microbiome organoid systems, brain organoids, and a robust interface of metabolites, neurons, and plasma in the near future will allow MGBA modeling for NDDs. Perhaps the most crucial step, i.e., the accumulation in various organs, takes time to standardize, but it will be a big leap in the in vitro disease modeling approach (Table 2, Fig. 3).

Transwell-based disease models

The Transwell model allows researchers to separate two types of cells using a semipermeable membrane while allowing chemical interaction and physical contact between them, as chemicals and released factors can pass through the membrane. Monolayering on either side of the membrane facilitates the formation of tight junctions, such as the BBB or epithelial barrier in the intestinal lumen [161] (Figs. 4 and 5).

One of the first robust Transwells to learn about host–microbiome interactions was by Ulluwishewa et al. [162]. Two workstations were developed in the Transwell setup. The insert was monolayered with Caco-2 cells on the membrane (apical facing the top and basal facing the membrane), and two media were used. The basal compartment was fed with an aerobic medium, and the insert, apical side, and anaerobic medium were used to culture *F. prausnitzii*. The system

Table 2 List of brain and intestinal organoids developed toward understanding MGBA

	Targeted model	Cell line/cell type	Type of organoid	Brief protocol	Key findings and uses	Limitations & use in MGBA modeling	References
1	Brain organoids for AD	iPSCs from patients with familial AD having duplicate APP or mutated PSEN	Scaffold-free 3D neural organoids with amyloid and tau pathology	12,000 iPSCs seeded in a 96-well plate, precoated with pluronic acid and Glasgow-MEM supplemented with KSR for 18–20 days; Transferred to adherent petri dish with medium to promote neuroepithelial formation for 15–20 days, after which heparin and Matrigel were added; Grown till Day 60	The spontaneous appearance of amyloid and tau proteins; Successfully studying the effect of β- and γ-secretase inhibitors	Complex steps and need for careful and skilled labor; Tissue necrosis after a time point, which can be improved with artificial BBB and microfluidics	[150]
2	Brain organoids for AD	iPSCs from familial AD patient groups	Cerebral organoids with cortical brain-like features	9000 cells/well seeded in ESC culture medium with ROCK inhibitor for six days; Medium changed to DMEM/F12 for another five to six days, after which the embryoid bodies were embedded in Geltrex in ultra-low attachment plate with DMEM/F12 and neurobasal medium for four to five days; Later transferred to differentiation medium and placed in an orbital shaker at 90 r/min	The expression and aggregation of amyloid and tau protein along with maturation over time being highly similar to AD pathology in vivo	The disease pathology being similar to only familial AD but not other mutations and phenotypes; Mature synaptic connections not observed in this model; Lack of vascularization, limiting the sustainability of the cells and long-term studies	[151, 152]
3	Brain organoids for AD	PITRM-1 knockout iPSCs	Cerebral organoids in Matrigel	20 days [141]	A relationship between mitochondrial protease (PITRM-1) deficiency and development of age dependent AD symptoms; Achieved mitochondrial clearance in iPSC-derived neurons	The model originally being made for a slow progressing disorder but giving spontaneous AD symptoms, which question the etiopathogenesis of the model and require further genetic and molecular understanding	[153]

Table 2 (continued)

	Targeted model	Cell line/cell type	Type of organoid	Brief protocol	Key findings and uses	Limitations & use in MGBA modeling	References
4	Brain organoids for PD	NESCs	Human midbrain differentiated on Matrigel with active synapses and electrophysiology	9000 NESCs seeded in low-attachment well plate in N2B27 medium for six days; Transferred to 24-well plate of ultra-low attachment with N2B27 medium for two more days; After eight days, colonies transferred on Matrigel droplet, kept in the same medium for another two days; Differentiation medium with neutrophic and growth factors added on Day 10; Placed in an orbital shaker on Day 14	High reproducibility of organoids up to 2 mm; Presence of neuromelanin (a primate brain protein); Robust and functioning neuronal cells	Absence of immune cells and vasculature, inhibiting the growth of organoids beyond a size; Necrotic center of organoids, leading to shrinkage and apoptosis of organoids	[154]
5	Brain organoids for PD	iPSCs from PBMCs	First organoid model of idiopathic form of PD	iPSCs in iPSC medium for four days for embryoid formation; Embryoid bodies transferred to V-shaped-well plate in DMEM/F12/neurobasal medium for four days; Medium changed to one with midbrain patterning factors for three days and Matrigel added with tissue growth induction medium for 24 h and transferred to nonadherent plate with differentiation medium while in an orbital shaker	Recreating the complicate dopaminergic neuronal network and expressing neuronal early and late markers that might be used for idiopathic PD; Presence of LMX1A and FOXA2 in the model, which are key progenitors for dopaminergic neurons; Higher expression of *PTX3* gene seen in PD-derived iPSCs	Multistep process with use of Sendai vector to form iPSCs from PBMC; No data about electrophysical activity of the dopaminergic neurons from PD-derived iPSCs and from normal cells	[155]

Table 2 (continued)

	Targeted model	Cell line/cell type	Type of organoid	Brief protocol	Key findings and uses	Limitations & use in MGBA modeling	References
6	Brain organoids for PD	hESCs	Midbrain-like organoids with electrophysiological function	10,000 hESCs/well in ultra-low attachment 96-well plate; Brain organoid generation medium added to the embryoid after 24 h; For mesencephalon, SMAD inhibitors used; After four days, growth factors added and, after another three days, embedded in Matrigel droplets; Later transferred to petri dishes containing brain organoid growth medium; After 48 h, moved to low attachment plates with maturation medium, and further placed in an orbital shaker for maturation	Fine mapping of WNT signaling and use of SMAD inhibitors determining the identity of midbrain-like organoids; The organoids producing dopamine and neuromelanin and being electrophysiologically functional	Not all types of cellular differentiation achievable even after long-term maturation; Low levels of standardization, efficiency, and reproducibility of these organoids	[156]
7	Intestinal organoids	Human iPSC lines	Intestinal organoid with enterocytes, Paneth cells, myoepithelial cells, and smooth muscle cells	Endoderm differentiation in RPMI 1640 medium with activin A for three days, followed by hindgut differentiation for four days with growth factors and WNT3A; Spheroids of age of three to four days embedded in Matrigel with growth factors for growth and differentiation for 21–100 days	The organoid having columnar epithelium mimicking the crypt-like villi; The organoid having enterocytes, Paneth cells, and enteroendocrine cells; Human intestinal stem cells also formed de novo during the hindgut development; Used to study drug absorption	More often used for gut developmental studies than MGBA studies for NDDs; Extremely complicated steps with careful medium preparation	[157]

Table 2 (continued)

	Targeted model	Cell line/cell type	Type of organoid	Brief protocol	Key findings and uses	Limitations & use in MGBA modeling	References
8	Intestinal organoids	Human iPSCs 253G1	HIOs	iPSCs differentiated into endoderm by incubation in RPMI 1640 with activin A and CHIR99021 for two hours; Hindgut differentiation in RPMI 1640 with B27, activin A, CHIR99021, and growth factors for four days; Floating spheroids transferred to Matrigel with intestinal growth medium for 14 days	Studying the effect of RA on differentiation of intestinal organoids; Overcoming the problem of a single phenotype Caco-2 monolayer; RA increasing the expression of CYP3A4 and tight junction protein ZO-1 and increasing TEER	No studies on host with microbiome; This model being used to study the intestine development and its microbiome on NDD etiology and occurrence rate	[158]
9	Intestinal organoids	hESC line H9 and nonpathogenic strain of *E.coli*	Immature intestinal organoids microinjected with *E.coli* ECOR2 and K-12 MG165	Passaged and differentiated as in [157]; The organoids maintained in medium with growth factors, Noggin, R-spondin, and Matrigel support; The formed organoids transplanted in mice *E.coli* and cultured in LB; Microinjection performed using thin wall glass capillaries	The integrity of the epithelial barrier being intact with fast *E.coli* growth and colonization; Colonization resulting in functional maturation of the epithelial barrier, with high regeneration of cells; The oxygen consumption and metabolite formation inside organoids changing gene expression and being able to stimulate mucus secretion	Intestinal microbial colonization being affected by the environment and diet of the individual patient, leading to the conclusion that *E.coli* is not the ideal culture; The entire functionality of intestine with immune cells and cellular maturation not achieved in this model	[159]

Table 2 (continued)

	Targeted model	Cell line/cell type	Type of organoid	Brief protocol	Key findings and uses	Limitations & use in MGBA modeling	References
10	Intestinal organoids	hESCs (H1, H9) and human iPSCs with PSC-derived NCC	HIO with ENS	hPSCs suspended in neural induction medium with RA added on Days 4 and 5; On Day 6, free floating spheroids embedded on fibronectin with the same medium without RA for four days; iPSCs grown in activin A for three days for endoderm formation; Growth factor and Chiron added on Day 5 to promote hindgut endoderm formation, which formed gut tube spheroids by Day 9; HIO and NCC brought together by low-speed centrifugation, embedded in Matrigel for four weeks, and maintained for eight weeks	NCC combined with HIO migrating in the mesenchymal layers and differentiating into glial cells and neurons; Showing neuronal activity; First model to incorporate functional neuronal network in intestinal organoid	The model used to study Hirschprung's disease, which can be extended to NDDs with changes in NCC formation	[160]

MGBA: microbiota–gut–brain axis; AD: Alzheimer's disease; PD: Parkinson's disease; PSC: pluripotent stem cell; iPSCs: induced PSCs; APP: amyloid precursor protein; PSEN: presenilin; PITRM-1: pitrilysin metallopeptidase 1; NESCs: neuroepithelial stem cells; PBMCs: peripheral blood mononuclear cells; hESCs: human embryonic stem cells; NCC: neural crest cell; 3D: three-dimensional; HIO: human intestinal organoid; ECOR2: escherichia coli restriction enzyme; K-12 MG165 *E. coli*: K-12 substr. MG1655; ENS: enteric nervous system; MEM: minimum essential medium; KSR: knockout serum replacement; ESC: embryonic stem cell; ROCK: rho-associated kinase; DMEM: Dulbecco's modified Eagle's medium; SMAD:suppressor of mothers against decapentaplegic; WNT: wingless-related integration site; CHIR99021: GSK3 inhibitor; LB: lysogeny broth; hPSCs: human PSCs; RA: retinoic acid; LMX1A: LIM homeobox transcription factor 1 alpha; FOXA2: forkhead box protein A2; *PTX3*: pentraxin-3; CYP3A4: cytochrome P450 3A4; Caco-2: cancer coli-2; ZO-1: zonula occludens-1; BBB: blood–brain barrier; NDDs: neurodegenerative diseases

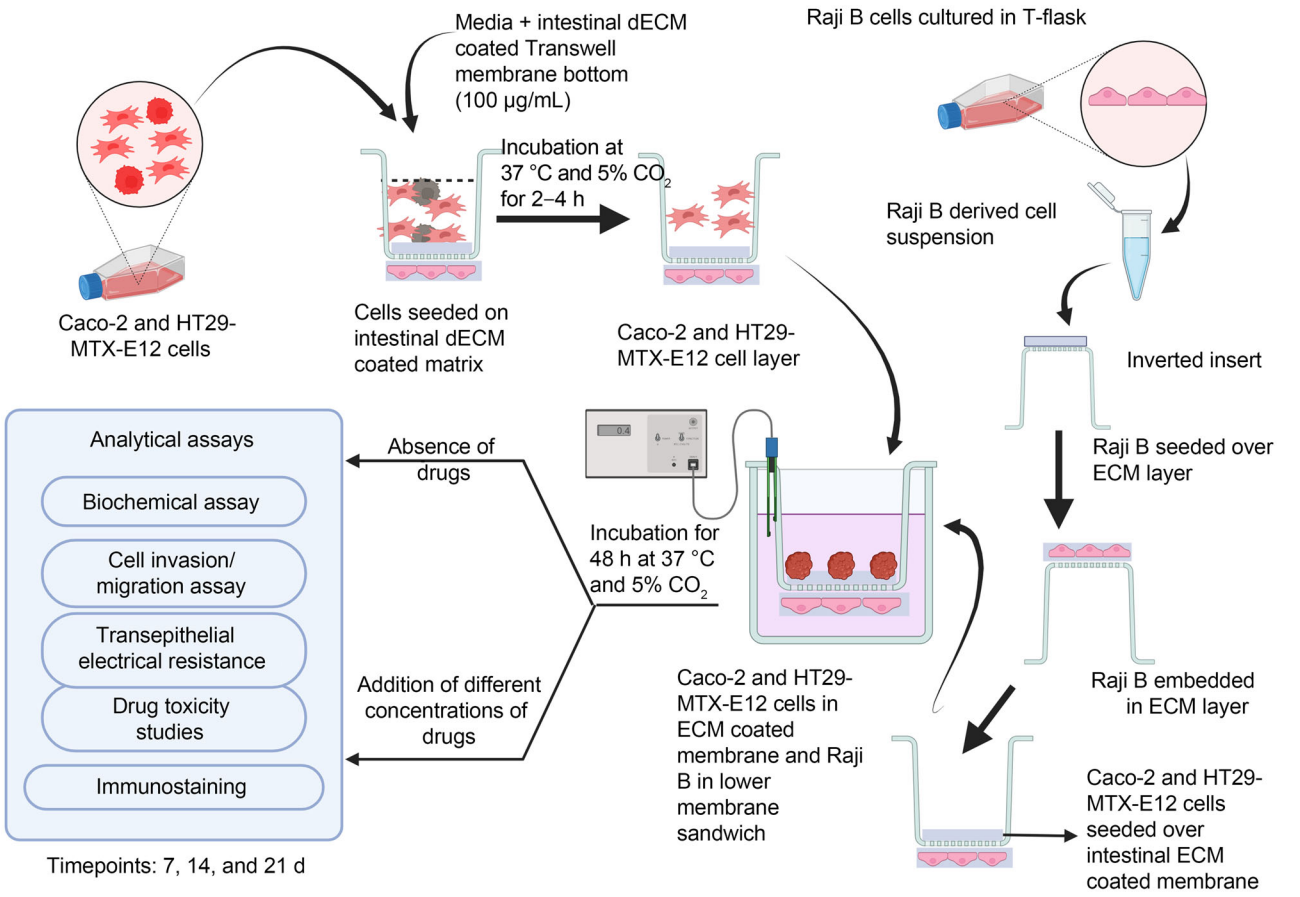


Fig. 4 Hypothesized development of the intestinal epithelial barrier. Schematic outline of Raji B-, HT-29-, and Caco-2-based 3D in vitro permeability model for BCS class II to IV drugs to identify the permeability enhancement, drug toxicity, and TEER. Caco-2: cancer coli-2; 3D: three-dimensional; BCS: biopharmaceutical classification system; TEER: transepithelial/transendothelial electrical; MTX: methotrexate; dECM: decellularized extracellular matrix

was covered with a lid. Gene expression showed low levels of inflammatory mediators in the insert; i.e., the apical side was exposed to *F. prausnitzii*. Thus, the species helps lower inflammation by producing anti-inflammatory compounds in the supernatant [162]. A similar method was used to study drug movement across Caco-2 monolayer cells on apical and basal sides but without aerobic or anaerobic compartments. A total of 3,000,000 Caco-2 cells were seeded on pre-wet filter inserts (12 mm in diameter). The basolateral chamber was filled with Dulbecco's modified Eagle's medium (DMEM). After 16 h of incubation, the apical medium was replaced with DMEM-PEST, which allowed the removal of nonadherent cells and reduced cellular aggregation to form multilayers. Special care was taken to ensure that monolayer integrity was not disturbed by the pipettes. Transepithelial/transendothelial electrical resistance (TEER) was measured before and after the permeability studies using an Endohm tissue resistance chamber, which checks the filter integrity. Permeability and active transport across the Caco-2 epithelial layer were measured by replacement experiments and by studying transporter proteins in the monolayer. Quality control of the Caco-2 monolayer was studied by confocal imaging of the morphology and immunohistochemical staining of proteins in tight junctions [163]. Caco-2 cells represent a single layer of the rate-limiting intestinal epithelium, which allows the study of drug diffusion across the intestinal barrier [164]. However, the Caco-2 cell line forms tighter junctions in the large intestine (colon) than in the small intestine. Hence, the paracellular movement of the drugs was restricted in this model. The intestinal barrier also consists of mucus-producing goblet cells, endocrine cells, and enterocytes, which aid digestion and absorption and are not replicated in a Caco-2 cell monolayer. They also overexpress glycoproteins that hinder their absorption [165]. Mini-guts or organoids were grown in intestinal tissue and cocultured with *Salmonella typhi* in a Transwell setup. Similar studies have been undertaken in which intestinal organoids were embedded at the top of the insert. Macrophages were allowed to adhere to the bottom of the insert. This setup

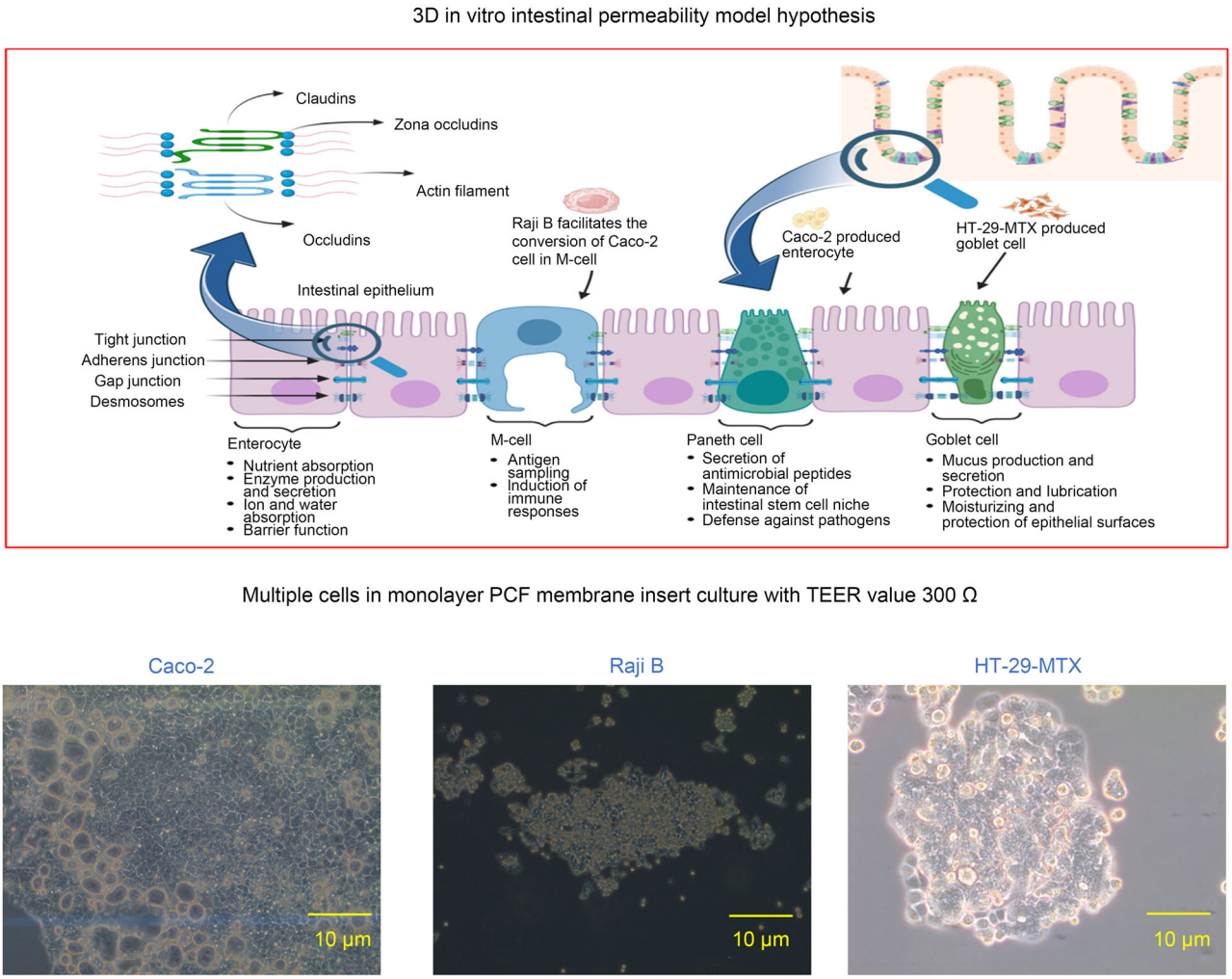


Fig. 5. 3D permeability model mechanism and cellular interplay. The gut epithelium is comprised of cells such as enterocytes, goblet cells, and Paneth cells that regulate the gut homeostasis. Coculture of Caco-2 cells with Raji B and HT-29-MTX is often used to mimic the whole microenvironment of the gut environment. Caco-2: cancer coli-2; MTX: methotrexate; PCF: phosphorus crystallization-filtration; TEER: transepithelial/transendothelial electrical resistance

provides the advantage of cells adhering to one another and being in close physical contact. *E. coli* infection changed the cytokine release pattern, and macrophages strengthened the barrier function, as evaluated by the increase in TEER and cell height. This was the first model for enteroid—macrophage coculture that also demonstrated gut physiology and immune reactions [145]. This model attempts to investigate the effects of immune cells in the host–microbiome model and explores the effect of the microbiome on maintaining a healthy immune system in the gastric environment and the effect of pathogenic strains on the equilibrium of the host–microbiome environment. However, cocultured macrophages need to be characterized by a functional phenotype and how they detect alterations in the microbiome and compartmentalize the release of different cytokines to maintain bodily homeostasis. The fact that it functions in close proximity to in vivo functions is a testament to its accuracy.

This model was taken a step further to introduce additional contributing factors, and a complicated model was developed by Lozoya-Agullo et al. They cocultured MTX-treated HT-29 cells, HT-29-MTX (matured mucus-producing cells), with Caco-2 monolayers and Raji B lymphocytes. Raji B cells acquire an M-cell microfold phenotype when grown with Caco-2 cells and perform functions such as transporting bacteria and viruses via endocytosis. This facilitates the understanding of the host–microbiome and immune system homeostasis. Caco-2 and HT-29-MTX cells were seeded in the apical chamber at a ratio of 90:10, and Raji B cells were seeded in the basolateral chamber. The permeability of 12 drugs was evaluated from the apical to basolateral direction, and the samples were analyzed by high-performance liquid chromatography. Results showed that the monolayer Caco-2 had tighter junctions and reduced permeability, whereas the triple culture was leaky due to the HT-29-MTX cells

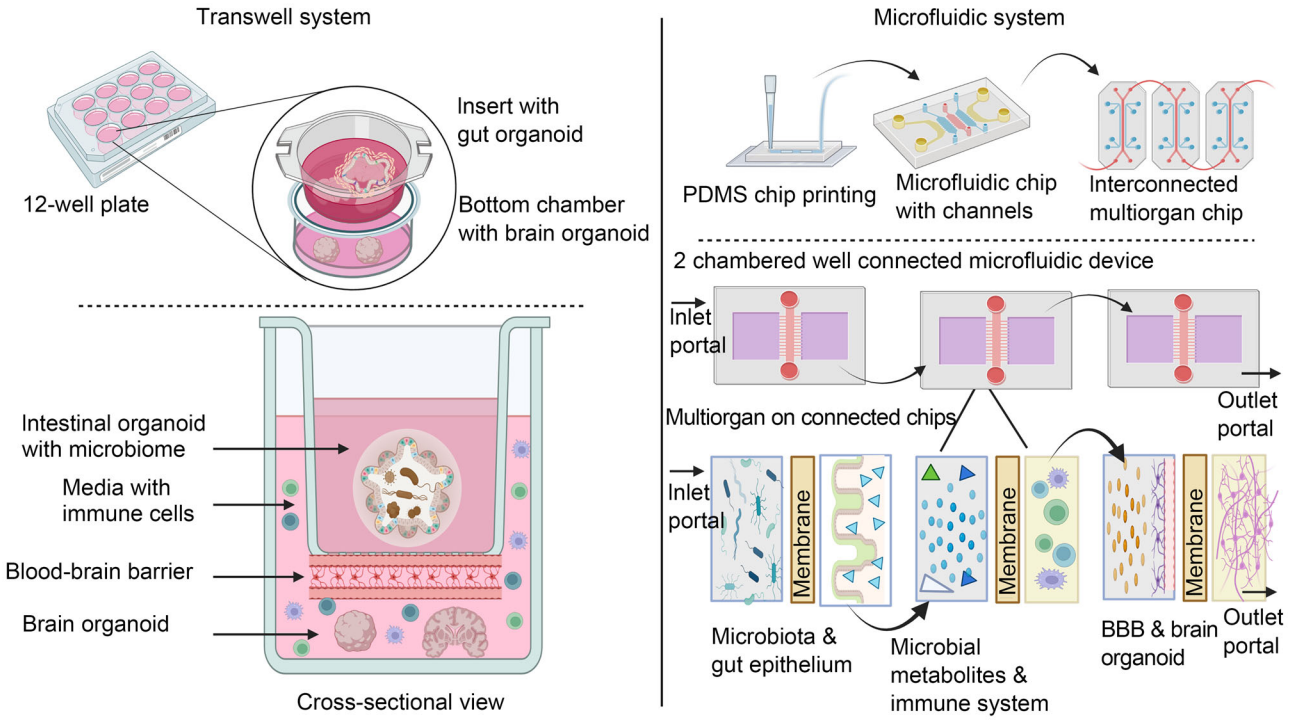


Fig. 6 3D in vitro modeling of MGBA with Transwell and microfluidic systems. The Transwell system involves two chambers separated by a semipermeable membrane, which allows the movement of chemical factors from one system to the other. Here, the apical chamber contains intestinal organoids with the microbiome, and the basolateral chamber has brain organoids. The medium in the well contains immune cells, which represent the interface of the gut and brain. The bottom compartment hosts a representative BBB between the two organ systems. A microfluidic system has the same separation as Transwell, but at a micro level, with a dynamic flow of nutrients. A two-chambered device can be used in connection to one another to recreate different interfaces like the microbiota and gut epithelium, the microbial metabolites and immune system, and finally the BBB and brain. 3D: three-dimensional; MGBA: microbiota–gut–brain axis; BBB: blood–brain barrier; PDMS: polydimethylsiloxane

in between. The coculture models were also similar to the rat small intestine obtained in situ. They successfully optimized the most physiologically relevant model, studied the permeability of drugs, and confirmed the classification using the biopharmaceutical classification system (BCS) criteria. However, because of the cost and complexity of optimizing the system, they suggested using a Caco-2 monolayer for initial drug screening and only later adding HT-29-MTX and Raji B cells to the model for oral drug absorption studies and a detailed understanding of transporters [166] (Fig. 6).

Transwell models can be limited as they consist of two chambers, whereas recapitulating an MGBA requires three separate systems: the gut, host–microbiome, and brain. It should also inculcate the two main barriers, the intestinal barrier and BBB, to separate the systems physically but stay connected via metabolites, immune cells, and signaling molecules. A roughly proposed idea could be using two Transwell inserts, with the two apical chambers representing the brain and gut, respectively. The basolateral chamber and media can be optimized to represent the circulation of signaling molecules and immune cells. The insert filter on the brain end can be lined to represent the BBB, whereas the gut can use intestinal organoids.

A step further to recreate the spatial orientation of MGBA is using the organ-on-chip concept that facilitates the control of multiple parameters at the micro-level. Despite working at a micro-level, they help understand cell interactions around variable physical cues like flow, shear stress, and strain. The flow also ensures that the microflora, metabolites, and signaling molecules travel from one compartment to another [167]. It also facilitates the movement of nutrient media, waste removal, and replacement or addition of specific factors, such as growth factors, inhibitors, or activators, to promote a particular process. Improvements in modeling include adding probes to check crucial parameters, such as O_2, pH, osmolarity, barrier integrity, and cell viability, eliminating the need for constant sampling and disturbing homeostasis [168]. A small setup size reduces the use of chemicals and space. Microfluidics has been used to model the BBB and intestinal epithelial cells. Integration of these barriers with vasculature-like channels will closely mimic in vivo organization. The human gut microbiome was recreated by Kim et al., which included two compartments separated by a porous membrane and lined by Caco-2 cells on ECM-coated walls that form intestinal tight junctions. Shear stress, cycling strain, and flow were maintained to mimic

Table 3 Transwell systems used to study gut epithelial barrier in MGBA

	List of the Transwell models	Intestine	Gut	Microbiota	Cell type	Hydrogel used	Antibody used	TEER ($\Omega \cdot cm^2$)	Time (d)	Screening parameters	References
1	3D model of the vertebrate gut	–	+	+	Caco-2, HT-29-MTX, IEC-monocyte, IEC-macrophage	Collagen	ZO-1, MUC2, IBA-1	Caco-2: 393.7±11.4; HT-29-MTX: 104.6±6.5; coculture of Caco-2 and HT-29-MTX: 204.2±6.5; IEC-monocyte: 250±12; IEC-macrophage: 300±43	21	Responses to parasite secretions in the presence of bacteria commensal to the gut	[175]
2	In vitro advanced barrier model	+	–	–	Caco-2, HT-29-MTX, Raji B	–	ZO-1, MUC2, MUC5 AC	Caco-2 monoculture: 396±63; Caco-2-Raji B coculture: 195±47; Caco-2/HT-29-MTX coculture: 334±44; Caco-2/HT-29-MTX/Raji B triculture: 297±53	22	SiO_2 nanoparticle translocation and uptake	[176]
3	In vitro intestinal epithelial cell model	+	–	–	Caco-2	–	–	Caco-2 monoculture: 1730±84	14	Transcytosis of *Bacillus subtilis* extracellular vesicles through an in vitro intestinal epithelial cell model	[177]
4	Triculture model of the small intestinal epithelium	+	–	–	Caco-2, HT-29-MTX, Raji B	–	–	Caco-2 monoculture: 2500	17	Toxicological effects of ingested nanocellulose	[178]

Table 3 (continued)

	List of the Transwell models	Intestine	Gut	Microbiota	Cell type	Hydrogel used	Antibody used	TEER ($\Omega \cdot cm^2$)	Time (d)	Screening parameters	References
5	Intestinal epithelial cell and mast cell coculture-based Transwell model	+	–	–	Caco-2, HT-29, HMC-1.2 cells	–	–	HT-29/Caco-2 coculture: 470.54±20.79; HT-29/Caco-2 coculture after the addition of HMC-1.2 cells: 470.54±20.79	21	Effect of the commercially available probiotic formulation; Serobioma against the LPS-induced intestinal inflammatory damage	[179]

Table 3 (continued)

	List of the Transwell models	Intestine	Gut	Microbiota	Cell type	Hydrogel used	Antibody used	TEER ($\Omega \cdot cm^2$)	Time (d)	Screening parameters	References
6	Caco-2 and monocyte-derived DC coculture-based Transwell model	–	+	–	Caco-2, monocyte-derived DCs	–	PE-conjugated anti-human CD1a, APC-conjugated anti-human CD83, PE-conjugated anti-human TGF-βRI/II/III, PE-conjugated anti-human human leucocyte antigen-DR, PE-conjugated anti-human CD86, PE-conjugated anti-human CD80, APC-conjugated anti-human CD40, PE-conjugated anti-human TSLPR	–	21	Mechanisms between the human enterocyte cell line, Caco-2, and the underlying human monocyte-derived DC in a Transwell model where Gram-positive (G^+) commensals prevent TLR4-dependent *E. coli*-induced semi-maturation in a TLR2-dependent fashion	[180]

Table 3 (continued)

	List of the Transwell models	Intestine	Gut	Microbiota	Cell type	Hydrogel used	Antibody used	TEER ($\Omega \cdot cm^2$)	Time (d)	Screening parameters	References
7	Inflammation-triggered "leaky gut" model	–	+	–	Caco-2, HT-29-MTX, THP-1	–	–	Caco-2 monoculture: 660±31; Caco-2/HT-29-MTX coculture: 605±29	21	Presence of mucus on the cell surface; increased intestinal permeability in Caco-2/HT-29-MTX-E12 coculture induced by IFN-γ priming	[181]
8	In vitro gut model	–	+	–	Caco-2, HT-29-MTX	–	Anti-Ep-CAM, anti-MUC2	Caco-2 monoculture: 510±60; HT-29-MTX monoculture: 230±40; Caco-2/HT-29-MTX coculture: 610±40	21	The permeability of the model as well as the material properties of the mucus produced by the model	[182]

Table 3 (continued)

	List of the Transwell models	Intestine	Gut	Microbiota	Cell type	Hydrogel used	Antibody used	TEER ($\Omega \cdot cm^2$)	Time (d)	Screening parameters	References
9	In vitro Transwell coculture of IECs and human intestinal-like DCs	+	–	+	Caco-2, human intestinal-like DCs	–	–	Caco-2 monoculture: 300	21	The presence of the live probiotic alone significantly increasing IL-1β, IL-6, IL-8, TGF-β2, RANTES, and IP-10 levels and decreasing IL-12p40, IL-10, TGF- β1, and MIP-1α levels; This release being correlated with a significant increase in the expression of almost all TLR signaling genes	[33]
10	Caco-2 Transwell model	+	–	–	Caco-2	–	–	Caco-2 monoculture: 1000	21	The absorption and transport mechanisms as well as the anti-inflammatory properties of ideain on Caco-2 Transwell model	[183]
11	Human enterocyte-like T84 cell Transwell model	+	–	+	T84	–	–	T84: 1850	12	Translocation of *Enterococcus faecalis* strains across a monolayer of polarized human enterocyte-like T84 cells	[184]
12	Human T84, HT-29, and HEK293T cell-based Transwell model	+	–	–	T84, HT-29, HEK293T cells	–	Anti-TCPTP CF-4, anti-ZO-1	VSL#3 (10^2, 10^4, and 10^6 CFU/mL, n=3) increasing TEER compared with untreated cells after 9- and 24-h treatment	20	VSL#3 reducing IFN-γ signaling and IFN-γ-induced epithelial barrier defects in a TCPTP-dependent manner	[185]

Table 3 (continued)

	List of the Transwell models	Intestine	Gut	Microbiota	Cell type	Hydrogel used	Antibody used	TEER ($\Omega \cdot cm^2$)	Time (d)	Screening parameters	References
13	Apical anaerobic model of the intestinal barrier	+	–	+	Caco-2	–	–	The mean TEER not differing between treatments at any time point, and the mean basal mannitol not differing between Caco-2 monolayers cocultured with *F. prausnitzii* or UV-killed *F. prausnitzii* at any time point	14	The interactions between commensal obligate anaerobes and intestinal epithelial cells	[162]
14	Caco-2/leucocyte coculture model	+	–	+	Caco-2, PBMC	–	–	Caco-2 with *E. coli* at 0 h: 620; Caco-2 with *E. coli* at 24 h: 583; Caco-2 with *Ljohnsonii* at 0 h: 591; Caco-2 with *Ljohnsonii* at 24 h: 425; Caco-2/PBMC with *E. coli* at 0 h: 632; Caco-2/PBMC with *E. coli* at 24 h: 549; Caco-2/PBMC with *Ljohnsonii* at 0 h: 598; Caco-2/PBMC with *Ljohnsonii* at 24 h: 478	21	The role of immune cells in the sensitization of human differentiated Caco-2 cells in recognizing signals originating from nonpathogenic bacteria	[186]

Table 3 (continued)

	List of the Transwell models	Intestine	Gut	Microbiota	Cell type	Hydrogel used	Antibody used	TEER ($\Omega \cdot cm^2$)	Time (d)	Screening parameters	References
15	In vitro 3D culture model of human intestinal epithelium	+	–	–	Caco-2, HT-29-MTX	Alginate, L-pNIPAM, L-pNIPAM-co-DMAc	CD10 antibody, ZO-1 antibody, Alkaline phosphatase antibody, Dipeptidyl peptidase IV antibody, Sucrase isomaltase antibody, MUC2 antibody, MUC5 AC antibody	–	21	3D culture and phenotypic marker expression of Caco-2 and HT-29-MTX cells on L-pNIPAM hydrogel scaffolds under dynamic culture conditions	[187]

MGBA: microbiota–gut–brain axis; TEER: transepithelial/transendothelial electrical resistance; 3D: three-dimensional; +: positive for 3D model; −: negative for 3D model; Caco-2: cancer coli-2; HT-29-MTX: methotrexate resistance HT-29 cell; IEC: intestinal epithelial cell; MUC2: mucin 2, oligomeric mucus/gel-forming; IBA-1: ionized calcium binding adaptor molecule 1; ZO-1: zonula occludens-1; DC: dendritic cell; HEK293T: human embryonic kidney 293; HMC-1.2: human mast cell line; THP-1: human monocytic cell line; PBMCL: peripheral blood mononuclear cell; L-pNIPAM: poly(N-isopropylacrylamide); L-pNIPAM-co-DMAc: laponite crosslinked, pNIPAM-DMAc copolymer; MUC5 AC: mucin 5AC, oligomeric mucus/gel-forming; PE: phycoerythrin; APC: allophycocyanin; TGF: transforming growth factor-β; TSLPR: thymic stromal lymphopoietin; Ep-CAM: epithelial Ca^{2+}-independent adhesion molecule; TCPTP: T-cell protein tyrosine phosphatase; UV: ultra-violet; LPS: lipopolysaccharide; TLR4: Toll-like receptor-4; TLR2: Toll-like receptor-2; IL: interleukin; IFN-γ: interferon-γ; RANTES: regulated on activation, normal T expressed and secreted; IP-10: interferon-γ inducible protein; MIP-1α: macrophage inflammatory protein

peristaltic motion [169]. Compared to the Transwell model, which is static and end-point-mediated, this gut-on-a-chip provides a live and dynamic understanding of the transport of molecules across the gut epithelium. Intestinal representations to study inflammation, immune regulation, and the effect of probiotics on gut microbes have been widely studied [170, 171]. The micro-BBB was characterized by Booth and Kim using bEnd.3 ECs cocultured with C8D1A astrocytes. Polydimethylsiloxane (PDMS) was used to construct the chip on a glass slide with two layers for electrodes and two for creating the channels, with a porous membrane to grow the cells and allow diffusion of signaling chemicals. Imaging, TEER measurement, and permeability studies showed that it was an optimum model to study changes in barrier function due to environmental stimuli, diseases, or drug response [172]. Similarly, the human brain EC line hCMEC/D3 was used, which can be cultured and used in microfluidic devices, with steady TEER values and expression of zonula occludens-1 (ZO-1; a tight junction protein expressed in the BBB and intestinal epithelial barrier). It could be used to study drug permeability; with some changes in working conditions, it could also be used for disease modeling [173].

To successfully model NDDs with the effect of the gut microbiome, an interface must be engineered to allow the passage of metabolites and secretions of the microbiome through bodily fluids, crossing the two main barriers and reaching the brain, and thus devising a more multiorgan-on-a-chip approach. The MINERVA platform, funded by the European Research Council, aims to develop this multiorgan approach. The success of this project will bridge the gap between neuroscience and the peripheral body [174].

In this extensive literature survey, there is still no Transwell system representing the gut, brain, microbiome, and plasma interface that connects the three systems. Some models study the gut and microbiome, NDDs, and barriers. This is a serious indication that optimal integration of the separate units should be tested to understand the hypothesis of the effects of the gut microbiome on brain disorders. The Transwell system offers great potential for MGBA development because it has two separate compartments: the gut and the brain. To a great extent, microfluidics has also helped devise these organ-on-a-chip models, although an ideal disease model has yet to be developed (Table 3).

Conclusions

Significant research has suggested that the gut microbiome and the brain are connected [188]. Communication via neurons, particularly the VN, has been highlighted in several animal models. Metabolites such as SCFAs also significantly impact the host's immune system and signaling. All pathways operating along the MGBA are varied and interconnected, as a metabolite from one can be a signal for another cross-communication. Although acceptance is somewhat controversial, there is abundant evidence that neurological disorders, such as ASD, PD, and AD, significantly affect the gut, resulting in brain–gut disorders like IBS. The opposite effect of the microbiome on the etiology of these diseases is yet to be confirmed. The variability in neurology and gut health in the population is a major hurdle, as specific connections cannot be concreted without doubt regarding genetics, dietary requirements, medication, environment, mental health, and upbringing.

Neural disorders have, at large, stifled researchers for a few reasons.

(i) Access to the brain or neurons is very dangerous.
(ii) Most neurological disorders have behavioral components that vary in patients.
(iii) Most therapeutics are rendered ineffective because xenobiotics do not cross the BBB.
(iv) Most medicines only treat symptoms, and the etiology and pathophysiology of these diseases are unknown.

Over many years of research, a new approach to studying neurodegenerative disorders via MGBA is promising. The emergence of biomolecular technologies and biomaterials has made it easier to facilitate replication of in vivo conditions in vitro. The emergence of Matrigel, hydrogels, and collagen scaffolds has allowed the recreation of the cell microenvironment in a laboratory. This has allowed the study of the diseases and therapies in laboratories much faster without the risk of ethical and social concerns. The discovery of stem cells and gene editing has allowed cell differentiation and growth control, enabling the production of specific cell types. Spheroid and organoid development is an important step toward more realistic disease models. Transwell systems still have the issue of being static; that is, they lack blood flow homeostasis. The disadvantages of in vitro models are their translatability and the ability to obtain a bigger picture. These studies were conducted on a small scale, so their effects may differ in animals or humans. It also requires time to standardize the model and achieve mass production. This also contradicts the need for in vitro models such as personalized disease models and therapies. The emergence of organ-on-a-chip has opened doors to generating multiorgan connections and a dynamic interplay between them.

There are challenges in developing disease models for neurological conditions owing to the aforementioned reasons, so there is still a long way to go. More research is needed to bring the brain and gut into the same model system with accurate physiological and molecular characteristics.

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Author contributions KA and LN collected the literature and wrote the manuscript. SM prepared the scientific illustrations and mechanism. SK and KK drafted the in vitro 3D intestinal barrier model. VR proofread and finalized the revised manuscript. SB and MS drafted and supervised the role of the neurological disease model. SR conceived the manuscript, prepared, and approved the final version of the manuscript.

Data availability The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study does not contain any studies with human or animal subjects performed by any of the authors.

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