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Integrating *in vitro* disease models of the neurovascular unit into discovery and development of neurotherapeutics

Yunfeng Ding[1], Eric V. Shusta[1,2] and Sean P. Palecek[1]

Abstract

The blood–brain barrier (BBB) regulates the transport of small molecules, proteins, and cells between the bloodstream and the central nervous system (CNS). Brain microvascular endothelial cells work with other resident brain cell types, including pericytes, astrocytes, neurons, and microglia, to form the neurovascular unit (NVU) and maintain BBB integrity. The restrictive barrier influences the pathogenesis of many CNS diseases and impedes the delivery of neurotherapeutics into the CNS. *In vitro* NVU models enable the discovery of complex cell–cell interactions involved in human BBB pathophysiology in diseases including Alzheimer's disease, Parkinson's disease, and viral infections of the brain. *In vitro* NVU models have also been deployed to study the delivery of neurotherapeutics across the BBB, including small molecule drugs, monoclonal antibodies, gene therapy vectors, and immune cells. The high scalability, accessibility, and phenotype fidelity of *in vitro* NVU models can facilitate the discovery and development of effective neurotherapeutics.

Addresses

[1] Department of Chemical and Biological Engineering, University of Wisconsin, Madison, WI, USA

[2] Department of Neurological Surgery, University of Wisconsin, Madison, WI, USA

Corresponding authors: Palecek, Sean P (sppalecek@wisc.edu); Shusta, Eric V (eshusta@wisc.edu)

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Neurovascular unit, Blood–brain barrier, Disease modeling, Neurotherapeutic, Drug delivery.

Abbreviations

BBB, blood–brain barrier; BMECs, brain microvascular endothelial cells; CNS, central nervous system; NVU, neurovascular unit; AD, Alzheimer's disease; PD, Parkinson's disease; CAR-T, chimeric antigen receptor T cell; hPSC, human pluripotent stem cell; iPSC, induced pluripotent stem cell; GBM, glioblastoma; mAb, monoclonal antibody; RMT, receptor-mediated transcytosis; scFv, single-chain variable fragment; AAV, Adeno-associated virus; Aβ, amyloid beta; CAA, cerebral amyloid angiopathy.

Introduction

The blood–brain barrier (BBB) is a dynamic interface that resides between the bloodstream and the central nervous system (CNS), facilitating CNS homeostasis. BBB properties are largely a consequence of brain endothelial cell characteristics that are regulated by other cellular components of the neurovascular unit (NVU), including pericytes embedded in the endothelial basement membrane, astrocytes, neurons, and perivascular macrophages [1]. In healthy individuals, the BBB plays a pivotal role in protecting the CNS from toxic substances via passive barriers and active transporters, limiting the transport of many small molecules, proteins, gene therapy vectors, and cells. However, in the event of CNS diseases, the BBB not only contributes to the pathogenesis of multiple CNS diseases but also continues to provide a significant barrier for the delivery of many neurotherapeutics. BBB dysfunction has been shown to play a central role in several CNS diseases, including multiple sclerosis, epilepsy, and stroke [2]. Although increased BBB permeability has also been observed in Alzheimer's disease (AD) and Parkinson's disease (PD), the role of the BBB in the pathogenesis of these neurodegenerative diseases is not yet clear [3,4]. The BBB impedes delivery of neurotherapeutics as a result of unique properties of brain microvascular endothelial cells (BMECs). BMECs express tight junction proteins, which form continuous networks of tight junction strands between adjacent BMECs that greatly reduce the paracellular transport of solutes. When compared with peripheral endothelial cells, BMECs also demonstrate lower levels of vesicular trafficking, which restricts transcellular transport of solutes. The polarized expression of efflux transporters, including P-glycoprotein, breast cancer resistance protein, multidrug resistance proteins, on the luminal (blood) side of BMECs further reduces the brain penetration of therapeutics that are substrates of these transporters (Reviewed in the study reported by Zlokovic [5]).

Recently, the emergence of a suite of new *in vitro* NVU models provides researchers with pivotal tools to systematically study both neurotherapeutic delivery and disease pathogenesis at the BBB in a well-defined, scalable fashion. *In vitro* NVU models are used to screen for BBB-crossing neurotherapeutics and to

identify strategies to improve neurotherapeutic transport across the BBB [6]. Incorporation of multiple NVU cell types in these models allows for simultaneous screening of BBB penetration and therapeutic efficacy, and permits elucidation of how different NVU components regulate molecular transport. Moreover, given the large species-to-species variation in BMEC transporter expression profiles, the ability to incorporate human cells in NVU models can help identify BBB delivery strategies that will translate to humans [7]. *In vitro* NVU models are also used to study the blood-to-brain and brain-to-blood trafficking of pathogenic substances, including toxic peptides and viruses, in disease modeling. Finally, *in vitro* NVU models can also be deployed to study the effects of disease-specific mutations on the integrity of the BBB. In this review, we explore the benefits and challenges of integrating *in vitro* NVU models into the discovery of CNS disease mechanisms and the development of neurotherapeutic delivery methods.

Neurotherapeutic delivery across the blood–brain barrier remains a significant hurdle

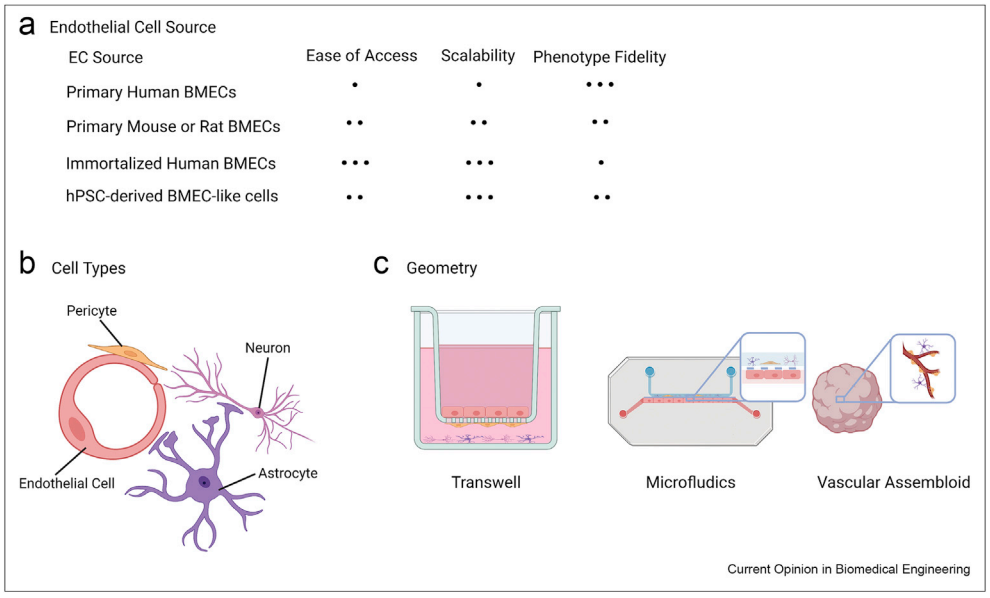
Although certain low molecular weight lipophilic molecules that are not substrates for efflux transport can efficiently cross the BBB and enter the CNS, most other therapeutics fail to cross the BBB and enter the brain in therapeutic amounts. Improving brain penetrance of new biologics and cell therapies to treat CNS disorders is crucial for their successful clinical implementation. For example, recent clinical trials evaluating gene therapy strategies to combat AD and PD all use invasive delivery strategies, such as intrathecal (NCT03186989, NCT03976349), intracisternal (NCT03634007, NCT04127578), and direct intracerebral delivery (NCT00876863, NCT03065192, NCT01621581) to bypass the BBB. The lack of an established human BBB-crossing gene therapy delivery system could therefore limit the clinical feasibility of CNS gene therapy. As another example, the BBB has limited the development of antibody therapies against CNS disease targets. Although intravenously-infused anti-amyloid monoclonal antibodies (mAbs) demonstrate promise in reducing amyloid load in transgenic AD mouse models, it is not clear whether the therapeutic effect can be translated to humans and whether brain antibody penetrance or the peripheral sink effect was the mechanism of action [8,9]. The extremely low level of brain penetrance of most therapeutic antibodies ($\sim$0.01% for the brain/plasma ratio [10]) contributes to the need for high antibody dosage and the termination of most AD antibody trials without successful outcomes [11]. The BBB can also be a significant hurdle in developing chimeric antigen receptor T cell (CAR-T) products against brain tumors. Because only a subpopulation of T cells can cross the BBB, penetrance of CAR-T products targeting brain tumors largely depends on BBB inflammation status and BBB disruption at the tumor sites, leading to variable treatment outcomes [12,13]. Thus, with the BBB being a key obstacle to the delivery of many therapeutics, it is essential to integrate brain penetration strategies into the development of neurotherapeutics. Meanwhile, the vast differences in BBB structure and function between healthy and diseased states call for the integration of both healthy and diseased NVU models into the neurotherapeutic development pipeline [2].

Cell sourcing and configurations of neurovascular unit models

In vitro NVU models differ in the types, sources, and geometric arrangement of NVU cells incorporated. Primary human and animal BMECs, immortalized human BMECs (e.g. human cortical microvessels endothelial cells/D3 (hCMEC/D3) cell line), and human pluripotent stem cell (hPSC)-derived BMEC-like cells are common endothelial cell sources. Recently, differences in gene expression between BMECs of human and other species have been described. Notably, the expressions of ATP-binding cassette (ABC) transporters, solute carrier (SLC) transporters, and several cell surface receptors were shown to be significantly different in BMECs isolated from different species (Reviewed in the study reported by Aday et al. [14]). As these transporters and surface receptors are involved in molecular trafficking and efflux activities, developing *in vitro* NVU models with human cells is likely to better predict molecular transport at the human BBB. Each of the endothelial cell sources has different advantages and disadvantages related to BBB phenotypes, ease of access, and scalability (Figure 1a). Although BMECs form the main interface encountered by therapeutics crossing from the bloodstream into the brain, other NVU cell types, including pericytes, astrocytes, and neurons, play a pivotal role in eliciting and regulating BBB characteristics in BMECs. These other NVU cell types are typically obtained from primary sources or differentiated from hPSCs. (Figure 1b, Reviewed in the study reported by Gastfriend et al. [15]).

A simple NVU model is the Transwell model, where BMECs are seeded onto a semipermeable membrane which divides the cell culture volume into a brain-mimicking lower chamber where non-endothelial NVU cell types are seeded and a blood-mimicking upper chamber (Figure 1c). The Transwell model provides a platform for rapidly screening bidirectional transport of therapeutics across a BMEC monolayer [6]. Because the Transwell system lacks physiologic flow which has been shown to affect BBB transport phenotypes, several microfluidic platforms now exist to incorporate fluid flow into BBB modeling while still permitting relatively straightforward assessment of permeability. Typically,

Figure 1



Summary of *in vitro* NVU models. **(a)** Common BMEC sources for *in vitro* NVU models and the general characteristics of these cell sources, including accessibility, scalability, and fidelity of the BBB phenotypes such as barrier formation and similarity of transporter expression profile to that of *in vivo* human BMECs, characterized as low (·), medium (··), or high (···). **(b)** Schematic of a cross-section of a brain capillary, showing the juxtaposition of an endothelial cell, pericyte, astrocyte, and neuron forming the NVU. **(c)** Common configurations of *in vitro* NVU models, including (i) Transwell model, (ii) microfluidic model, and (iii) vascular assembloid model. BBB, blood–brain barrier; BMEC, brain microvascular endothelial cells; NVU, neurovascular unit.

BMECs are seeded in one microfluidic channel, whereas other NVU cell types are seeded in a neighboring channel separated by a hydrogel layer or a semipermeable membrane (Reviewed in the study reported by Katt and Shusta [16]). Although microfluidic models often incorporate more intimate cell contact and shear stress modeling than that provided by Transwell systems, there have also been efforts to better recapitulate the three-dimensional (3D) arrangement of NVU cell types in a microfluidic device. Notably, Vatine et al. [17] assembled hPSC-derived BMEC-like cells into a hollow vessel-like structure in one channel and seeded hPSC-derived neural progenitors in a neighboring channel separated by a laminin-coated, porous flexible PDMS membrane, highlighting a more accurate NVU morphology. NVU assembloid models have also been described recently. For example, a spheroid model with primary human astrocytes at the core and primary or immortalized brain endothelial cells and pericytes encasing the spheroid has been developed to identify brain-penetrating agents [18]. Moreover, Blanchard et al. [19] reconstructed a human NVU assembloid model for disease modeling. Induced pluripotent stem cell (iPSC)-derived BMEC-like cells were encapsulated in Matrigel with pericytes and astrocytes, leading to the formation of a stable vascular-like network inside the assembloid. Although assembloid NVU models can mimic aspects of the *in vivo* NVU structure, quantifying molecular transport in these 3D assembloids can be significantly more complex than in Transwell or microfluidic models.

In vitro NVU models can facilitate development of better delivery methods to cross the blood–brain barrier

Given the significant hurdle of therapeutic delivery across the BBB and recent advancements in NVU modeling, researchers have incorporated *in vitro* NVU models into the identification, development, and analysis of strategies for molecular and cellular delivery across the BBB (Table 1).

Small molecule drugs

In vitro NVU models are useful for studying mechanisms of drug uptake and transport at the BBB. They also enable researchers to predict therapeutic penetrance of the BBB and develop delivery strategies in an *in vitro* model before moving to animal testing. For example, the blood vessel-like microfluidic model described in the previous section was used to determine the relative permeability of colchicine, retigabine, and levetiracetam, suggesting that microfluidic NVU models can be used to predict human CNS drug penetrance [17]. *In vitro* Transwell NVU models seeded with iPSC-derived BMEC-like cells have also been shown to successfully predict permeabilities of small molecule drugs in several studies [20–22]. *In vitro* NVU models can also be used to design BBB-crossing

Table 1

Development and analysis of neurotherapeutic delivery strategies using *in vitro* NVU models.

	Delivery strategy	*In vitro* NVU model	Major findings	References
Small molecule drugs	Drug only (gabapentin)	hCMEC/D3	Gabapentin is a substrate for the influx transporter LAT1 at therapeutic concentrations.	[51]
	Drug only (colchicine, retigabine, and levetiracetam)	iPSC-derived BMEC-like cells, 3D microfluidic model	The model exhibited physiologically relevant transendothelial electrical resistance and accurately predicted blood-to-brain permeability of pharmacologics.	[17]
	Drug only (atenolol, cimetidine, prazosin, trazodone, caffeine, hydroxyzine, propranolol, donepezil, memantine, galantamine, and rivastigmine)	iPSC-derived BMEC-like cells, Transwell	iPSC-derived NVU Transwell model exhibits an *in vivo*-like phenotype. After passing a benchmarking analysis, such model can be used to evaluate brain permeabilities of drugs.	[20]
	Drug only (diazepam, caffeine, ibuprofen, celecoxib, diclofenac, loratadine, and rhodamine 123)	iPSC-derived BMEC-like cells, Transwell	The iPSC-derived NVU Transwell model demonstrated expected drug permeabilities *in vitro* and is thus suitable for drug transport studies.	[21]
	Drug only (befloxatone, flumazenil, raclopride, erlotinib, verapamil, buprenorphine, 2F-A85380-tartrate, loperamide, dextromethorphan, levofloxacin, sulfasalazine, caffeine, and taurocholate)	iPSC-derived BMEC-like cells, Transwell	There is a good correlation between *in vitro* and *in vivo* drug brain permeability. An iPSC-derived BBB model can be integrated into CNS drug screening.	[22]
	PLGA nanoparticles loaded with elvitegravir	Mouse brain endothelial cells, Transwell	Elvitegravir nanoformulation demonstrated an improved BBB model penetration and an enhanced HIV-1 suppression in infected human monocyte-derived macrophages after crossing the BBB model.	[24]
	Liposomes with ApoE-derived peptides loaded with drug	Rat brain endothelial cells, Transwell	The permeability of a tritiated curcumin derivative was enhanced after its entrapment into ApoE-nanoliposomes.	[23]
	GBM-targeting peptide-conjugated Ferri-liposome loaded with doxorubicin	Mouse brain endothelial cells, Transwell	The GBM-targeting peptide-conjugated Ferri-liposome was rapidly transported across an *in vitro* BBB model and displayed GBM-specific cellular uptake and doxorubicin release.	[25]
Monoclonal antibodies	Apoferritin-conjugated monoclonal antibody	hCMEC/D3, Transwell	Apoferritin-conjugated monoclonal antibody can be imported by hCMEC/D3 cells and released on the 'brain' side of the model.	[26]
	Basigin monoclonal antibody	hCMEC/D3, Transwell	mAbs binding to the basigin receptor can internalize into human brain endothelial cells and cross the BBB using RMT.	[27]
	46.1-scFv monoclonal antibody	iPSC-derived BMEC-like cells, Transwell	By screening a nonimmune, human single-chain variable fragment (scFv) phage display	[28]

Table 1 (*continued*)

	Delivery strategy	*In vitro* NVU model	Major findings	References
			library on a human iPSC-derived BMEC-like cell Transwell model, antibodies such as the 46.1-scFv that exhibited increased transport across the BBB were identified.	
	Transferrin receptor monoclonal antibody	hCMEC/D3, Transwell	The ability of antibodies to the transferrin receptor to cross the BBB was determined by their relative affinities at different extracellular and endosomal pH levels.	[52]
	Brain shuttle peptide-conjugated anti-amyloid monoclonal antibody	hCMEC/D3, Transwell	Brain shuttle peptide-conjugated anti-amyloid antibody had increased transcellular transport across the BBB when compared with the parent antibody.	[29]
	Antibody-triggered RMT	iPSC-derived BMEC-like cells, Transwell	This hPSC-derived *in vitro* BBB model discriminated species-selective antibody-mediated transcytosis mechanisms and was predictive of *in vivo* CNS exposure of rodent cross-reactive antibodies.	[30]
Adeno-associated virus	AAV9	Primary human BMEC, Transwell	AAV9 penetrated human BMEC barriers more effectively than AAV2 but had reduced transduction efficiency.	[39]
	AAV-BR1	hCMEC/D3	AAV-BR1 was identified as a capsid variant with high specificity and high transduction efficiency for BMECs.	[42]
	Shuttle peptide-enhanced AAV8	hCMEC/D3, Transwell	BBB shuttle peptide-enhanced AAV8 demonstrated an increased ability to cross the BBB and a higher transduction in the brain after a systematic administration.	[41]
Cells	CD4+ T cells	Hematopoietic stem cell-derived BLEC model	Under non-inflammatory conditions Th1* and Th1 CD4+ T cells preferentially crossed the BBB. Under inflammatory conditions, the migration rate of all Th subsets across the BBB was comparable.	[13,53]
	CD4+ T cells	Mouse brain endothelial cells bEnd.5	The *in vitro* BBB model supported T cell adhesion under static and physiological flow conditions.	[54]
	Peripheral blood monocyte-derived macrophages	Primary rat brain BMEC	Misfolded tau protein increased expression of ICAM-1, VCAM-1 and selectins at the endothelium, facilitating blood-to-brain cell transmigration.	[55]
	CD4+ T cells	iPSC-derived BMEC-like cells	An iPSC-derived BBB model that displayed an adhesion molecule phenotype suitable for immune cell interaction was developed.	[45]

BBB, blood–brain barrier; BMEC, brain microvascular endothelial cells; CNS, central nervous system; hPSC, human pluripotent stem cell; iPSC, induced pluripotent stem cell; mAb, monoclonal antibody; NVU, neurovascular unit; 3D, three-dimensional; RMT, receptor-mediated transcytosis.

nanocarriers. For example, a rat brain endothelial cell-derived Transwell model was used to show that nanoliposomes covalently coupled with ApoE-derived peptides have increased blood-to-brain transport of a tritiated curcumin derivative [23]. Using the bEnd.3 mouse brain endothelial cell line in a Transwell model, Gong et al. [24] demonstrated an improvement in BBB penetration of the antiviral drug elvitegravir when using a poloxamer poly(lactic-co-glycolic acid) nanoformulation. A similar Transwell model with the addition of glioblastoma cells in the 'brain' chamber was used to demonstrate the ability of glioblastoma-targeting peptide-conjugated Ferri-liposomes to cross the BBB and deliver doxorubicin to glioblastoma cells, leading to suppression of tumor cell proliferation [25]. This study highlights the ability to simultaneously test BBB penetration and analyze therapeutic efficacy in a single *in vitro* NVU model.

Monoclonal antibodies

Efforts to increase antibody delivery across the BBB have focused on fusing therapeutic mAbs to antibody or peptide moieties that target endogenous BBB transporters, thereby facilitating brain uptake of the conjugated therapeutic cargo. *In vitro* NVU models are important for developing and refining such strategies. Using immortalized human BMECs in a Transwell configuration, H-ferritin nanoparticles were found to facilitate the delivery of mAbs across the BBB by transferrin receptor-mediated transcytosis (RMT) [26]. A similar model revealed that mAbs bound to the basigin receptor were able to cross the BBB via RMT [27]. Using an iPSC-derived *in vitro* NVU model, Georgieva et al. [28] screened a nonimmune, human single-chain variable fragment phage display library on a human iPSC-derived BMEC-like cell model and identified antibodies that exhibited increased transport across the BBB. NVU models may also be used to predict brain penetration capabilities of therapeutic mAbs fused to BBB-crossing peptides or bispecific antibodies that can bind both a therapeutic target and an RMT receptor for enhanced delivery [29,30]. Notably, a Transwell model seeded with iPSC-derived BMEC-like cells was found to be able to accurately predict *in vivo* CNS exposure of rodent cross-reactive antibodies using *in vitro* permeabilities of these antibodies in the NVU model [30].

Adeno-associated virus

The ability to engineer recombinant adeno-associated virus (AAV) particles to deliver genes that encode therapeutic proteins has made AAV vectors the safest and the most popular option for gene therapies [31]. The search for an AAV vector that efficiently crosses the BBB and transduces target CNS populations after intravenous (IV) administration is an ongoing challenge. Deverman et al. [32] reported that AAV-PHP.B, an AAV variant selected via Cre-dependent AAV targeted evolution, can cross the BBB and transduce adult mouse CNS cells efficiently after IV injection. Subsequently, LY6A was identified to be the receptor on BMECs responsible for AAV-PHP.B transport [33,34]. Although this vector has shown promising results for delivery of therapeutic genes to treat genetic CNS disease in mouse models [35,36], AAV-PHP.B only transits the BBB in a selected subset of mice strains and fails to significantly transduce rhesus CNS cells after IV injection [37,38]. Thus, the field is still seeking AAV variants that efficiently cross the human BBB. *In vitro* NVU models using human BMECs may enable efforts to identify and engineer AAV vectors for human BBB transport. For example, AAV9 was found to traffic through a primary human BMEC Transwell model more efficiently than AAV2 in one study [39,40]. Zhang et al. [41] found that AAV8, when fused to a transferrin receptor-binding shuttle peptide, demonstrated increased transcytosis across an hCMEC/D3-seeded Transwell model. BMECs themselves may also be a target for gene therapy. AAV-BR1, an AAV2 mutant identified in an *in vivo* screening of random ligand libraries displayed on AAV capsids, can selectively transduce mouse brain endothelial cells *in vivo* and the hCMEC/D3 human immortalized BMEC line *in vitro* [42]. Given the large species-to-species differences of BMEC transporter expression profiles, performing screening in human cell-based *in vitro* NVU models could lead to newly engineered AAV capsids with human BBB-crossing capabilities [7].

Immune cells

With the emergence of engineered immune cell products against cancers, the development of CAR-T and other cellular immunotherapies to treat brain tumors is underway [43]. However, the transmigration of immune cells across the BBB is highly limited in healthy individuals but can occur under inflammatory conditions associated with CNS disease or injury [44]. Transwell-based NVU models with immune cells seeded in the apical chamber have been used to model immune cell transmigration across the BBB, leading to new knowledge on the mechanism of immune cell trafficking at the BBB. Notably, Nishihara et al. [13] found that Th1 and Th* subsets of CD4+ T cells preferentially crossed the endothelial monolayer in a human *in vitro* NVU model under non-inflammatory conditions. Furthermore, the recent development of an hPSC-derived NVU model for immune cell interaction studies allows for the combination of hPSC-derived BMEC-like cells and autologous immune cells in a single *in vitro* NVU model, highlighting the possibility for patient-specific disease modeling [45]. As cell-based therapies advance to treat CNS disorders, *in vitro* NVU models will play an important part in engineering strategies to direct the cells to the appropriate CNS compartments.

Table 2

Uses of *in vitro* NVU models to study CNS disease pathogenesis.

Disease	*In vitro* NVU Model	Major findings	References
Alzheimer's disease	Microfluidic NVU model with the hCMEC/D3 and control or AD ReN human neural progenitor cell cells	BBB dysfunctions were present in AD models, including increased permeability, reduced tight junction protein expression, increased MMP2 and reactive oxygen species, and deposition of amyloid at the vascular endothelium.	[46]
	Isogenic APOE3 and APOE4 iPSC-derived vascular organoid model	Dysregulation of calcineurin–NFAT signaling and APOE in pericyte-like mural cells induced APOE4-associated pathology.	[19]
	Three-dimensional bioengineered vessel NVU model	ApoE and HDL synergized to facilitate Aβ transport across the endothelial cell barrier. ApoE4 was less efficient than ApoE2 in promoting Aβ transport.	[56]
	iPSC-derived Transwell NVU model	Aβ and neuroinflammation signals increased IgG uptake and transport in the model.	[57]
	Mouse brain endothelial cell Transwell model	$A\beta_{1\text{-}42}$ induced tight junction damage and BBB leakage in the bEnd.3 Transwell model. RAGE played an important role in the process.	[58]
	Primary mouse brain capillary endothelial cell Transwell model	Basolateral recombinant ApoJ and apical ApoA1 facilitated the transport of basolateral $A\beta_{1\text{-}40}$ in this model.	[59]
	Rat brain microvascular endothelial cells	Exposure to $A\beta_{25\text{-}35}$ disrupted tight junctions, increased BBB permeability, decreased cell viability in this model.	[60]
	hCMEC/D3 Transwell model	hCMEC/D3 cells had limited utility in studying Aβ trafficking owing to the low barrier tightness of this model.	[61]
Parkinson's disease	Microfluidic NVU model with iPSC-dopaminergic neurons, iPSC-derived BMEC-like cells, and primary human astrocytes, microglia, and pericytes	α-synuclein fibril treatment induced key aspects of Parkinson's disease phenotypes, including accumulation of pSer129-αSyn, mitochondrial impairment, neuroinflammation, and increased BBB permeability.	[48]
	hCMEC/D3 Transwell model	α-synuclein preformed fibrils impaired tight junction protein expression in endothelial cells.	[47]
	Rat cerebral microvessel endothelial cells and C6 astroglial cells Transwell model	PD drug FLZ was effluxed by P-gp in rat cerebral microvessel endothelial cells.	[62]
	Primary rat brain endothelial cells and pericytes Transwell model	Pericytes were more sensitive to monomeric α-synuclein than endothelial cells regarding release of inflammatory cytokines and MMP-9 in this model.	[63]
Viral infection	Microfluidic NVU model with hCMEC/D3 cells	SARS-CoV-2 spike protein S1 triggered a proinflammatory response and promotes loss of barrier integrity in this model.	[49]
	iPSC-pericyte-like-cell-containing cortical organoid	Pericyte-like cells integrated into the cortical organoid were infected with SARS-CoV-2 and served as viral replication hubs.	[64]
	hCMEC/D3 Transwell model	Zika virus infected hCMEC/D3 cells without disrupting BBB permeability and tight junction protein expression, and the virus was subsequently released on the brain side.	[65]
	iPSC-derived Transwell NVU model	Zika virus infected iPSC-derived BMEC-like cells without disrupting BBB permeability and tight junction protein expression and was subsequently released on the brain side.	[50]
	Primary human brain microvascular endothelial cells Transwell model	West Nile virus infected primary human BMECs, leading to increased permeability, increased leukocyte adhesion, and transmigration across the *in vitro* model.	[66]

Aβ, beta-amyloid; BBB, blood–brain barrier; BMEC, brain microvascular endothelial cell; CNS, central nervous system; iPSC, induced pluripotent stem cell; NVU, neurovascular unit; P-gp, P glycoprotein; APOE3, apolipoprotein E isoform ε3; APOE4, apolipoprotein E isoform ε4; MMP2, matrix metalloproteinase-2; IgG, immunoglobulin G; RAGE, receptor for advanced glycation endproducts; ApoJ, apolipoprotein J; ApoA1, apolipoprotein A1; hCMEC/D3, human cortical microvessels endothelial cells/D3; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

Using *in vitro* neurovascular unit disease models to study central nervous system disease pathogenesis

NVU dysfunction has been implicated in the pathogenesis of numerous CNS diseases, including AD, PD, and viral infection [2]. *In vitro* NVU models can be purposed for disease modeling, helping to elucidate molecular mechanisms underlying the role of the BBB in these CNS diseases, and facilitating the development of new neurotherapeutics (Table 2).

Alzheimer's disease

AD is a neurodegenerative disease with amyloid beta (Aβ) deposition in the CNS being one of the most significant pathological hallmarks. Although BBB dysfunction can be observed in patients with AD, there is not a consensus about whether it contributes to early AD pathogenesis. The construction of a 3D microfluidic NVU model incorporating hCMEC/D3 cells and control or AD ReN human neural progenitor cells revealed that AD neural progenitor cells were sufficient to induce increased BBB permeability, decreased expression of tight junction proteins, and deposition of Aβ at the endothelium, successfully recapitulating the BBB damage of AD *in vitro* [46]. Recently, Blanchard et al. constructed a vascular assembloid model for cerebral amyloid angiopathy, a disease sharing the amyloid deposition pathology with AD, using isogenic *APOE3* or *APOE4* human iPSC-derived BMEC-like cells, mural cells, and astrocytes. Increased Aβ deposition was identified in assembloids containing *APOE4* mural cells. The vascular assembloid model recapitulates amyloid pathologies of cerebral amyloid angiopathy *in vitro*, indicating that the NVU in AD is affected by the *APOE4* allele that carries higher AD risk and that the NVU might play a role in *APOE4*-linked AD pathogenesis [19].

Parkinson's disease

PD is a neurodegenerative disease with the abnormal accumulation and aggregation of α-synuclein in the CNS being one of the most significant pathological hallmarks. Recently, researchers found that BBB leakage can be observed among PD patients [4]. In an hCMEC/D3 Transwell model, α-synuclein preformed fibrils reduced the expression of tight junction proteins ZO-1 and occludin in the hCMEC/D3 cells, suggesting that BBB damage could be a result of PD pathologies [47]. Notably, Pediaditakis et al. assembled a microfluidic NVU model containing human iPSC-derived dopaminergic neurons, human iPSC-derived BMEC-like cells, and human primary brain astrocytes, microglia, and pericytes. Exposure of the model to α-synuclein fibrils induced key PD phenotypes in this model, including accumulation of pSer129-αSyn, neuroinflammation, and increased BBB permeability [48]. This multicellular model recapitulated α-synuclein toxicity in multiple NVU cell types and demonstrated that α-synuclein can directly disrupt the BBB.

Viral infection

Most pathogens fail to cross the BBB. However, viruses such as SARS-CoV-2, Zika virus, and West Nile virus have been reported to infect neural cells and cause neuropathologies. The mechanisms these viruses use to enter the CNS remain unknown, with BBB penetration being one likely route. In a microfluidic-based NVU model containing hCMEC/D3 cells, SARS-CoV-2 spike protein S1 triggered a pro-inflammatory response and promoted loss of endothelial barrier integrity [49]. The model recapitulated the response of the NVU to luminal SARS-CoV-2 exposure, potentially explaining the observation of neurological complications in a subset of patients with COVID-19. Moreover, by exposing iPSC-derived BMEC-like cells in a Transwell NVU model to Zika virus in the luminal 'blood' chamber, it was found that Zika virus infected iPSC-derived BMEC-like cells without disrupting BBB permeability and tight junction protein expression, and the virus was subsequently released into the 'brain' side [50]. This model helped elucidate the mechanism by which Zika virus crosses the BBB and gains access to the CNS.

Conclusions and future directions

The emergence of *in vitro* NVU models provides a platform that enables and accelerates BBB screening, design, and optimization components of neurotherapeutic development. Improved NVU models, particularly those of human origin, can be powerful complementary tools for modeling the effects of disease on the BBB. A variety of *in vitro* NVU models exist, with different cell types, cell sources, and model configurations. The choice of the appropriate *in vitro* NVU model depends on the application. As human BBB model fidelity improves, we anticipate more applications integrating *in vitro* human NVU models into the discovery and development process of neurotherapeutics. For example, the use of human *in vitro* NVU models enables screening of neurotherapeutic delivery strategies, including BBB-crossing peptide-decorated liposomes, bispecific mAbs targeting both the NVU for delivery and the CNS target for therapeutics, and engineered AAV capsids that are able to cross the BBB and transduce neurons and astrocytes. We also anticipate a broader application of *in vitro* NVU models to study CNS disease pathogenesis. For example, by assembling *in vitro* models using NVU cell types differentiated from patient-specific control and disease human iPSC lines, researchers can study disease pathogenesis in a personalized fashion. Given the species differences in both transport at the BBB and CNS disease mechanisms, the incorporation of human cells into *in vitro* NVU models is of particular importance. In the future, *in vitro* NVU models may also be incorporated into the manufacturing

process of neurotherapeutics, enabling measurement of BBB permeability as a release criterion in the quality control process. We envision that given the relatively low cost and complexity of some of the *in vitro* NVU models, samples of BBB-crossing therapeutics could be tested for penetrance in these models to ensure efficacy and minimize batch-to-batch variability.

Author contributions

Yunfeng Ding: Conceptualization, Writing — Original draft preparation. **Eric V. Shusta**: Conceptualization, Writing — Review and Editing, Supervision: **Sean P. Palecek**: Conceptualization, Writing — Review and Editing, Supervision.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The authors have multiple issued patents and patent applications in the field of BBB modeling.

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Figure 1 was created with BioRender.com.

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