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In Vitro Blood-Brain Barrier Models: Method Development, Validation and Application in CNS Drug Permeability Assessment

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ABSTRACT

The blood-brain barrier (BBB) represents one of the most significant challenges in central nervous system (CNS) drug development, severely limiting therapeutic access to the brain. In vitro BBB models have emerged as indispensable tools for understanding barrier physiology and assessing drug permeability, offering alternatives to costly and ethically complex animal studies. This review examines the evolution of BBB modeling techniques, from simple monolayer cultures to sophisticated three-dimensional neurovascular unit reconstructions. Method development has progressed from immortalized cell lines like bEnd3 and hCMEC/D3 to primary brain endothelial cell cultures and advanced co-culture systems incorporating astrocytes, pericytes, and neurons. These models demonstrate enhanced barrier properties, including elevated transendothelial electrical resistance and reduced permeability coefficients, more closely mimicking in vivo conditions. Validation approaches encompass morphological assessments, functional barrier integrity measurements, and transporter activity evaluations using established reference compounds. Contemporary applications span high-throughput screening platforms for pharmaceutical discovery, mechanistic studies of transporter-mediated drug movement, and pathophysiological modeling of neuroinflammatory conditions. Emerging technologies, including microfluidic organs-on-chips and human induced pluripotent stem cell-derived models, promise improved physiological relevance and personalized medicine applications. Despite significant advances, current models face limitations in fully recapitulating the complex three-dimensional architecture and hemodynamic forces of the neurovascular unit. Nevertheless, these in vitro systems continue to provide valuable insights into BBB biology and serve as essential tools for CNS drug development, bridging the gap between molecular understanding and clinical translation.

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Introduction

The blood-brain barrier (BBB) represents one of the most restrictive biological interfaces in the human body, functioning as a selective gatekeeper that maintains central nervous system (CNS) homeostasis by regulating molecular transport between systemic circulation and brain parenchyma [1]. This specialized endothelial barrier poses significant challenges for pharmaceutical development, as it prevents approximately 98% of small-molecule drugs and nearly all large-molecule therapeutics from reaching therapeutic concentrations in the brain [2]. Consequently, CNS diseases remain among the most challenging therapeutic targets, with limited treatment options available for conditions such as Alzheimer's disease, Parkinson's disease, and brain tumors.

The development of reliable *in vitro* BBB models has emerged as a critical tool for understanding barrier function and screening CNS drug candidates. These models aim to recapitulate the complex architecture and physiological properties of the native BBB, including tight junction formation, specialized transport mechanisms, and metabolic functions [3]. Traditional *in vitro* approaches have evolved from simple cell culture systems to sophisticated three-dimensional models incorporating multiple cell types and dynamic culture conditions, reflecting the growing understanding of BBB complexity and the need for more physiologically relevant screening platforms. Contemporary *in vitro* BBB models encompass several methodological approaches, including static transwell systems, dynamic microfluidic devices, and organoid-based platforms.

The validation of these models relies on multiple complementary techniques, with transendothelial electrical resistance (TEER) measurements serving as the gold standard for assessing barrier integrity. TEER values are strong indicators of the integrity of the cellular barriers and provide a quantitative assessment of tight junction formation [4]. Additional validation parameters include measurement of paracellular permeability using molecular tracers, expression of tight junction proteins (claudin-5, occludin, ZO-1), and functional assessment of efflux transporters such as P-glycoprotein.

Recent advances in stem cell technology have revolutionized BBB modeling through the development of human induced pluripotent stem cell (iPSC)-derived brain microvascular endothelial cells. These systems offer significant advantages over immortalized cell lines, including human-specific physiology, patient-specific disease modeling capabilities, and improved predictive accuracy for drug permeability. Our findings showed a good correlation between *in vitro* iPSC-based models and *in vivo* human brain permeability data, demonstrating the translational potential of these advanced systems [5]. The application of *in vitro* BBB models in CNS drug development spans multiple stages, from early compound screening to mechanistic studies of drug transport. These models enable high-throughput assessment of drug permeability, identification of transport mechanisms, and evaluation of drug-drug interactions at the BBB. Furthermore, they provide platforms for studying BBB dysfunction in disease

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states and testing therapeutic interventions targeting barrier restoration [6]. Next-generation in vitro models of the BBB should consider zonation, brain region, age, sex, ethnicity, and disease state specificity, reflecting the need for more sophisticated and personalized approaches to BBB modeling. Despite significant advances, current *in vitro* BBB models face ongoing challenges, including incomplete barrier tightness compared to *in vivo* conditions, lack of physiological flow dynamics, and limited representation of the neurovascular unit complexity. Future developments focus on incorporating additional cell types (pericytes, astrocytes, neurons), implementing physiological flow conditions, and developing disease-specific models that better recapitulate pathological BBB dysfunction. The integration of advanced analytical techniques, including real-time monitoring systems and multi-omics approaches, promises to further enhance the predictive power and mechanistic insights provided by these essential drug development tools.

Overview of Blood-Brain Barrier Structure and Function and Method Development of *In Vitro* BBB Models

Overview of Blood-Brain Barrier Structure and Function

Cellular Components of the BBB

The blood-brain barrier represents a highly specialized neurovascular unit comprising multiple cellular components that work synergistically to maintain cerebral homeostasis. The BBB is formed by endothelial cells of the capillary wall, astrocyte end-feet ensheathing the capillary, and pericytes embedded in the capillary basement membrane, along with the capillary basement membrane itself [7].

Brain microvascular endothelial cells (BMECs) form the primary structural component of the BBB, creating a continuous monolayer connected by specialized tight junctions. These endothelial cells differ significantly from peripheral endothelial cells, exhibiting unique characteristics including the absence of fenestrations, minimal pinocytotic activity, and expression of specialized transport systems [8]. The BBB is structurally composed of a layer of brain capillary endothelial cells bound to each other through tight junctions, with their development, maintenance, and properties controlled by other brain cells that contact the BCECs: pericytes and glial cells.

Pericytes are contractile cells embedded within the capillary basement membrane, constituting approximately 20-30% of capillary length in brain microvasculature. Pericytes help control blood flow and remove toxins, while also playing crucial roles in BBB development, maintenance, and regulation of vascular permeability [9]. These cells contribute to basement membrane production and provide structural support to the microvasculature.

Astrocytes, the most abundant glial cells in the brain, extend specialized processes called end-feet that completely surround brain capillaries. Astrocytic endfeet help keep the balance of ions and provide essential paracrine signals that maintain BBB integrity. Astrocytes secrete factors such as glial cell line-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), and transforming growth factor-β (TGF-β) that promote tight junction formation and barrier function [20]. Figure shows the structure of Blood blood-brain barrier.

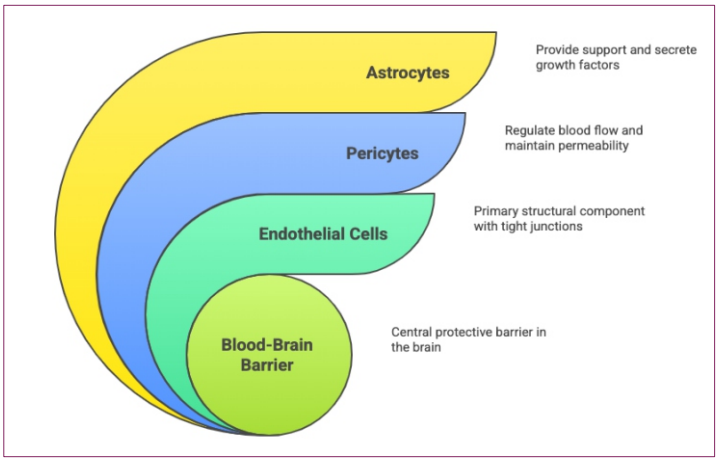

Fig 1: Blood Brain Barrier structure

Key Barrier Properties Relevant to Drug Permeability

The BBB's restrictive nature stems from several key properties that collectively limit drug penetration into the brain. Tight junctions between endothelial cells create a high-resistance paracellular pathway, with transendothelial electrical resistance (TEER) values *in vivo* reaching 1500-8000 Ω·cm^2, significantly higher than peripheral tissues [11]. These tight junctions are composed of transmembrane proteins, including claudins, occludins, and junctional adhesion molecules (JAMs), anchored to the cytoskeleton through zonula occludens proteins.

The BBB exhibits highly selective transcellular transport mechanisms, including specific transporters for essential nutrients (glucose transporter GLUT1, amino acid transporters LAT1 and LAT2) and efflux pumps that actively remove xenobiotics from the brain. P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and multidrug resistance proteins (MRPs) constitute the primary efflux systems that limit drug accumulation in the brain [12].

Method Development of *In Vitro* BBB Models

Types of *In Vitro* BBB Models

2D Monolayer Models

Primary Brain Microvascular Endothelial Cells (BMECs)

Primary BMECs represent the most physiologically relevant cell source for BBB modeling, as they retain species-specific characteristics and native transporter expression profiles. The first in vitro BBB model was constructed in 1974 using cerebral cortex endothelial cells of cows cultured on nylon sieves, with the first in vitro model with human brain cells established in 1985. Primary cells are typically isolated from brain microvessels using density gradient centrifugation and enzymatic digestion protocols. However, primary brain cells often show batch-to-batch variability, loss of barrier function during *ex vivo* culture, and very low transendothelial electrical resistance (TEER), a measure of paracellular barrier integrity. Bovine brain microvascular endothelial cells (BBMECs) have been extensively used due to their relative availability and ability to form moderately tight barriers, though they require careful culture conditions to maintain barrier properties [13].

Immortalized Cell Lines

Immortalized cell lines offer advantages of reproducibility, unlimited proliferation capacity, and reduced batch-to-batch variation compared to primary cells.

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Several immortalized human brain capillary endothelial cell lines have been developed, including hCMEC/D3, hBMEC, TY10, and BB19, for establishing improved human *in vitro* BBB models suitable for high-throughput screening. The hCMEC/D3 cell line, derived from human temporal lobe microvessels, represents one of the most widely used immortalized models. These cells express characteristic BBB markers, including claudin-5, occludin, and VE-cadherin, along with functional P-glycoprotein efflux activity. However, TEER values typically remain below 50 $\Omega \cdot cm^2$, significantly lower than *in vivo* values [14]. The RBE4 cell line, derived from rat brain endothelial cells, exhibits relatively high TEER values (up to 150 $\Omega \cdot cm^2$) and maintains expression of tight junction proteins and efflux transporters. Similarly, the b.End3 and b.End5 mouse brain endothelial cell lines provide reproducible barrier models, though with species-specific limitations for human drug development applications [15].

Co-culture Models

Endothelial Cells with Astrocytes and/or Pericytes

Co-culture systems aim to recapitulate the multicellular environment of the BBB by incorporating supportive cell types that enhance barrier function. BBB models include brain microvascular endothelial cell monolayer models, BMEC-astrocyte coculture models, and BMEC-pericyte-astrocyte triple-culture models. Astrocyte co-cultures significantly enhance endothelial barrier properties through paracrine signaling mechanisms. The most common co-culture configuration involves endothelial cells cultured on the upper surface of transwell inserts with astrocytes seeded on the lower surface or bottom of the culture well. This setup allows for paracrine communication while maintaining spatial separation. Studies have demonstrated that astrocyte-conditioned medium can increase TEER values by 2-3 fold compared to monoculture conditions [16]. Pericyte co-cultures have gained increasing attention due to their role in BBB development and maintenance. Triple co-culture models incorporating endothelial cells, astrocytes, and pericytes have shown improved barrier tightness and more physiologically relevant gene expression profiles compared to simpler systems [17].

3D and Organoid Models

Spheroids, Hydrogels, and Organoids

Three-dimensional BBB models aim to better recapitulate the complex architecture and cellular interactions of the neurovascular unit. Spheroid models involve the formation of multicellular aggregates containing endothelial cells, astrocytes, and pericytes in suspension culture. These models allow for enhanced cell-cell contact and matrix deposition compared to traditional 2D systems. Hydrogel-based models incorporate endothelial cells within three-dimensional matrices such as collagen, Matrigel, or synthetic hydrogels. These systems enable the formation of tubular structures that more closely resemble native capillary architecture. Microfluidic systems include human induced pluripotent stem cell-derived endothelial cells, brain pericytes, and astrocytes as self-assembled vascular networks in fibrin gel, with gene expression of membrane transporters, tight junction, and extracellular matrix proteins consistent with native tissue. Organoid models represent the most advanced 3D approach, generating brain-like tissue structures containing multiple cell types in physiologically relevant spatial arrangements. These models can incorporate patient-specific iPSC-derived cells, enabling personalized disease modeling and drug screening applications.

Dynamic and Microfluidic Models

Dynamic *In Vitro* BBB (DIV-BBB)

Dynamic models introduce physiological flow conditions that more accurately represent *in vivo* hemodynamics. Dynamic *in vitro* BBB models incorporate flow-based systems that apply shear stress to cultured endothelial cells, promoting barrier maturation and maintenance. The cone-plate apparatus represents one approach for applying controlled shear stress to BBB cultures. Hollow fiber-based systems represent another dynamic approach, with endothelial cells cultured on the inner surface of hollow fibers while culture medium flows through the fiber lumen. This configuration allows for continuous perfusion and waste removal while maintaining barrier function [18].

Microfluidic and Synthetic Microvasculature Models

Microfluidic BBB models offer precise control over cellular microenvironments, enabling the creation of perfused vascular networks with physiologically relevant dimensions. These systems typically incorporate parallel channels separated by porous membranes, allowing for independent perfusion of vascular and brain compartments. Recent advances in microfluidic technology have enabled the creation of organ-on-chip models that incorporate multiple cell types, flowing medium, and mechanical forces. These systems can model BBB dysfunction in disease states and evaluate therapeutic interventions under controlled conditions [19]. Figure 2 demonstrates the development of *in vitro* BBB models.

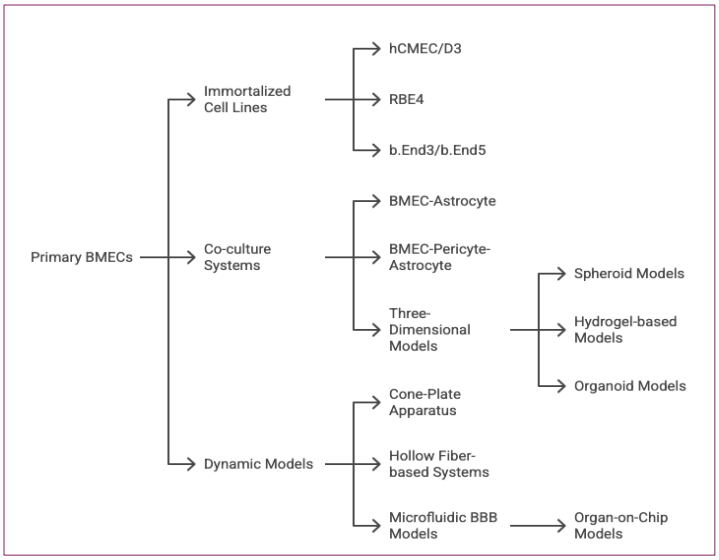

Figure 2: Development of in vitro BBB models

Important Considerations in Model Development

Cell Source and Species

The choice of cell source significantly impacts model relevance and translational potential. Human cell sources provide the highest clinical relevance but may be limited by availability and technical challenges. Human cells may be needed when human-specific transporters/receptors or immunologic issues are involved, though primary human cells are not usually available because of ethical reasons. Species differences in transporter expression, tight junction proteins, and metabolic pathways must be carefully considered when selecting cell sources. Rodent models may not accurately predict human drug permeability due to species-specific differences in efflux transporter expression and activity.

Culture Conditions and Barrier Induction

Optimal culture conditions are critical for developing and

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maintaining barrier function *in vitro*. Factors including medium composition, serum concentration, growth factors, and substrate coating significantly influence barrier development. Serum-free or low-serum conditions often promote tighter barrier formation, while specific growth factors such as basic FGF and retinoic acid can enhance barrier properties. The use of astrocyte-conditioned medium or co-culture systems provides essential paracrine factors that promote barrier induction and maintenance. Hydrocortisone supplementation has been shown to enhance tight junction formation and increase TEER values in several BBB models [20].

Measurement of Barrier Integrity

TEER and Permeability Assays

TEER measurements provide a quantitative assessment of barrier integrity, with high junctional tightness characterized by TEER averaging almost 800 Ω cm^2, though TEER values of 100-300 Ω cm^2 are more commonly reported in studies on mouse primary endothelial cells. TEER measurements should be performed using appropriate electrode configurations and standardized protocols to ensure reproducibility. Permeability assays using molecular tracers of different sizes and properties provide complementary information about barrier function. Commonly used tracers include sucrose (paracellular permeability), mannitol (paracellular), and fluorescein (transcellular). Transport studies should include both passive diffusion and active efflux assessments.

Reproducibility and Scalability

Reproducibility represents a critical challenge in BBB modeling, particularly for primary cell-based systems. Standardized protocols, quality control measures, and inter-laboratory validation studies are essential for ensuring consistent results. The development of Good Cell Culture Practice (GCCP) guidelines specific to BBB models would enhance reproducibility across research groups. Scalability considerations include the ability to perform high-throughput screening applications while maintaining model relevance. Automated culture systems and standardized assay protocols can facilitate larger-scale studies, though careful validation is required to ensure that scaling does not compromise model quality.

Validation of *In Vitro* BBB Models

Criteria for Validation

Morphological and Molecular Marker Expression

The validation of *in vitro* BBB models requires a comprehensive assessment of morphological characteristics and molecular marker expression that reflect the native BBB phenotype. Tight junction proteins of the blood-brain barrier are vital for maintaining the integrity of endothelial cells lining brain blood vessels, serving as primary validation criteria for model authenticity.

Essential tight junction proteins include claudin-5, occludin, and zonula occludens (ZO) proteins [21]. Assessments of tight junction proteins occludin, claudin 5, and scaffold proteins ZO1 and ZO2 in endothelial cells form the foundation of BBB validation [21]. Claudin-5 represents the most BBB-specific tight junction protein, demonstrating restricted expression in brain endothelial cells and playing a crucial role in maintaining paracellular barrier function (Cecchelli et al., 2007). Occludin, while not BBB-specific, contributes significantly to barrier tightness and serves as an important validation marker [22].

Morphological validation includes assessment of endothelial cell monolayer formation, presence of intercellular tight junctions visualized through immunofluorescence microscopy, and absence of fenestrations characteristic of peripheral endothelium [23]. Ultrastructural examination using transmission electron microscopy provides detailed visualization of tight junction morphology and basement membrane structure [24]. Additional molecular markers include adherens junction proteins (VE-cadherin, β-catenin), basement membrane components (laminin, collagen IV), and BBB-specific transporters [25]. The expression of glucose transporter 1 (GLUT1) serves as a key validation marker, as it is highly expressed in brain endothelial cells and essential for glucose transport across the BBB [26].

Functional Assessment

Functional validation encompasses evaluation of barrier integrity, transporter activity, and metabolic function [22]. Transendothelial electrical resistance (TEER) measurement provides a quantitative assessment of paracellular barrier tightness. The BBB is formed by endothelial cells with special characteristics, which confer the BBB with low permeability and high transendothelial electrical resistance (TEER) [27]. Valid BBB models should demonstrate TEER values approaching physiological levels, typically exceeding 150 Ω·cm^2 for acceptable models.

Permeability assessment using molecular tracers of varying sizes validates paracellular barrier function. Commonly employed tracers include sucrose (342 Da), mannitol (182 Da), and fluorescein isothiocyanate-dextran (various molecular weights). Low permeability coefficients for these hydrophilic tracers confirm effective paracellular barrier formation.

Efflux transporter validation involves assessment of P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and multidrug resistance proteins (MRPs) functionality. Bidirectional transport studies using specific substrates and inhibitors demonstrate active efflux. For instance, rhodamine 123 transport studies with verapamil inhibition validate P-gp functionality, while prazosin transport assesses BCRP activity. Influx transporter validation includes assessment of nutrient transporters such as GLUT1 for glucose, large amino acid transporter 1 (LAT1) for amino acids, and monocarboxylate transporter 1 (MCT1) for lactate and ketones. Functional validation requires demonstration of saturable, competitive transport kinetics characteristic of carrier-mediated transport.

Comparison with *In Vivo* Data

Validation requires a systematic comparison of *in vitro* model characteristics with published *in vivo* BBB properties. TEER values, permeability coefficients, and transporter expression levels should align with reported *in vivo* measurements. Species-specific differences must be considered when comparing human cell-based models with rodent *in vivo* data. Protein expression profiling using quantitative proteomics or Western blotting validates the relative abundance of key BBB proteins. Transcriptomic analysis provides additional validation through assessment of gene expression patterns characteristic of brain endothelial cells versus peripheral endothelium.

Correlation with *In Vivo* Permeability

Use of Clinical or Preclinical Data for Benchmarking

Establishing correlations between *in vitro* permeability

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measurements and *in vivo* BBB penetration represents a critical validation step for model utility in drug development. A study of twenty drugs shows similar compound ranking between rat and human models, although with a 2-fold higher permeability in rat, with an *in vitro/in vivo* correlation in rat ($R^2 = 0.67$; $P = 2 \times 10^4$) highlighted.

Clinical positron emission tomography (PET) data provide a gold standard benchmarking for human BBB permeability. A very good correlation ($r^2 = 0.90$; $P < 0.001$) was demonstrated between *in vitro* BBB permeability and *in vivo* permeability coefficient using clinical PET radioligands, establishing the predictive validity of well-characterized *in vitro* models.

Preclinical benchmarking utilizes established CNS and non-CNS drugs with known brain penetration profiles. The bovine brain microvessel endothelial cell (BBMEC) apparent permeability coefficient (P_app) correlation with *in vivo* BBB penetration using microdialysis sampling demonstrates the utility of comparative studies for model validation.

Cerebrospinal fluid (CSF) drug concentrations provide additional benchmarking data, though the relationship between CSF and brain interstitial fluid concentrations varies depending on drug properties and elimination pathways. Brain tissue homogenate drug concentrations offer direct assessment of brain penetration but require careful consideration of brain binding and distribution factors.

Case Studies Demonstrating Predictive Value for CNS Drug Penetration

Several case studies have demonstrated the predictive value of validated *in vitro* BBB models for CNS drug development. Findings showed a good correlation between the *in vitro* iPSC-based human BBB model and *in vivo* human brain permeability measurements using clinical PET radioligands, validating the translational potential of advanced *in vitro* systems.

Caffeine, a well-characterized CNS-penetrant compound, demonstrates high permeability in validated BBB models consistent with its rapid brain penetration and psychoactive effects. Conversely, mannitol shows minimal permeability in validated models, reflecting its exclusion from brain tissue and utility as a paracellular permeability marker.

Morphine and its metabolite morphine-6-glucuronide provide an instructive comparison, with morphine showing higher BBB permeability than its hydrophilic metabolite, consistent with their differential CNS pharmacological effects. P-glycoprotein substrates such as loperamide demonstrate low brain penetration in validated models despite high lipophilicity, reflecting efflux transporter activity. The antidepressant fluoxetine shows moderate permeability in validated BBB models, consistent with its therapeutic CNS effects and plasma-brain concentration ratios. Dopamine demonstrates minimal permeability due to its hydrophilic nature, validating the need for the prodrug approach used with L-DOPA for Parkinson's disease treatment. Validation studies have confirmed the utility of *in vitro* BBB models for predicting the brain penetration of novel CNS drug candidates, with successful correlation between *in vitro* permeability measurements and subsequent clinical brain exposure data. These case studies support the integration of validated *in vitro* BBB models into early-stage CNS drug development pipelines.

cLogP< 5, PSA < 120 Å, MW < 450 were confirmed as essential for CNS drugs, demonstrating how validated BBB models can confirm established medicinal chemistry principles for brain-penetrant drug design.

The predictive value of these models continues to improve with advancing cell culture technologies and validation methodologies.

Application in CNS Drug Permeability Assessment

Screening and Selection of CNS Drug Candidates

High-throughput screening applications

In vitro blood-brain barrier models have revolutionized CNS drug discovery through high-throughput screening platforms that enable rapid assessment of drug permeability. Cell-based assays using immortalized brain endothelial cell lines such as hCMEC/D3 and bEnd.3. Provide standardized platforms for screening large compound libraries [28]. These models allow pharmaceutical companies to evaluate hundreds of compounds simultaneously, measuring transendothelial electrical resistance (TEER) and permeability coefficients to identify promising CNS drug candidates early in the development process [29].

Lead optimization and structure-permeability relationships

Primary cultures of brain endothelial cells and co-culture systems with astrocytes have proven invaluable for establishing structure-activity relationships (SAR) in CNS drug development. These models help medicinal chemists understand how molecular modifications affect BBB penetration, enabling rational drug design. The parallel artificial membrane permeability assay for the blood-brain barrier (PAMPA-BBB) has emerged as a cost-effective tool for lead optimization, providing rapid permeability predictions that correlate well with *in vivo* brain uptake [30].

Mechanistic Studies

Transporter-mediated drug movement

In vitro BBB models have been instrumental in elucidating transporter-mediated drug transport mechanisms. These systems enable researchers to study the role of efflux transporters like P-glycoprotein and BCRP in limiting CNS drug penetration [31]. Co-culture models incorporating pericytes and astrocytes better recapitulate the neurovascular unit, allowing investigation of transporter regulation and drug-transporter interactions under physiologically relevant conditions.

Pathophysiological modeling

Advanced *in vitro* models have enabled the study of BBB dysfunction in disease states. Inflammatory conditions can be modeled by exposing endothelial monolayers to pro-inflammatory cytokines like TNF-α and IL-1β, demonstrating increased permeability and altered transporter expression [12]. These pathophysiological models provide insights into how neuroinflammation affects drug delivery and help identify therapeutic targets for CNS disorders.

Limitations and Future Directions

Gaps in recapitulating in vivo complexity

Despite significant advances, current *in vitro* BBB models face limitations in fully recapitulating the complexity of the neurovascular unit. Many cell-based models lack the three-dimensional architecture and hemodynamic forces present *in vivo*, potentially leading to overestimation of drug permeability [9]. Additionally, species differences between human and animal cell lines can complicate the translation of findings to clinical applications.

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Emerging technologies and model improvements

Microfluidic "organs-on-chips" technology represents a promising advancement, incorporating fluid flow and mechanical forces to better mimic physiological conditions [13]. Integration of human induced pluripotent stem cell-derived brain endothelial cells offers potential for personalized medicine approaches and improved human relevance in drug screening applications [12].

Conclusion

In vitro blood-brain barrier models have fundamentally transformed CNS drug discovery by providing accessible, reproducible platforms for studying barrier physiology and drug permeability. The progression from simple cell monolayers to sophisticated neurovascular unit reconstructions demonstrates the field's commitment to improving physiological relevance while maintaining experimental practicality. The integration of multiple cell types, three-dimensional architectures, and dynamic culture conditions has significantly enhanced model fidelity, enabling more accurate predictions of *in vivo* drug behavior. High-throughput screening applications have accelerated compound evaluation, while mechanistic studies have elucidated fundamental transport processes and pathophysiological changes in disease states. However, current limitations underscore the need for continued innovation. The complexity of the neurovascular unit, with its intricate cellular interactions and hemodynamic forces, remains challenging to fully recapitulate in vitro. Future developments in microfluidic technology, human stem cell biology, and tissue engineering hold promise for addressing these limitations. The field's trajectory toward more physiologically relevant models, combined with advances in analytical techniques and computational modeling, positions *in vitro* BBB systems as increasingly powerful tools for CNS drug development. As these models continue to evolve, they will likely play an even more central role in translating fundamental neuroscience discoveries into therapeutic interventions for neurological disorders.

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Conflict of Interest Statement

The authors declare that there are no commercial or financial relationships that could be construed as a potential conflict of interest. All authors confirm that the research was conducted in the absence of any relationships or circumstances that could be perceived to influence the objectivity or integrity of the work presented.

Informed Consent: Not applicable

Ethical Approval: Not applicable

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