Drug Development for Central Nervous System Diseases Using In vitro Blood-brain Barrier Models and Drug Repositioning

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Abstract: An important goal of biomedical research is to translate basic research findings into practical clinical implementation. Despite the advances in the technology used in drug discovery, the development of drugs for central nervous system diseases remains challenging. The failure rate for new drugs targeting important central nervous system diseases is high compared to most other areas of drug discovery. The main reason for the failure is the poor penetration efficacy across the blood-brain barrier. The blood-brain barrier represents the bottleneck in central nervous system drug development and is the most important factor limiting the future growth of neurotherapeutics. Meanwhile, drug repositioning has been becoming increasingly popular and it seems a promising field in central nervous system drug development. In vitro blood-brain barrier models with high predictability are expected for drug development and drug repositioning. In this review, the recent progress of in vitro BBB models and the drug repositioning for central nervous system diseases will be discussed.

Keywords: Blood-brain barrier, central nervous system disease, drug development, drug repositioning, neurotherapeutics, penetration efficacy.

1. INTRODUCTION

An important goal of biomedical research is to translate basic research findings into useful medical advances. Despite the advances in the technology used in drug discovery, the development of drugs for the central nervous system (CNS) diseases remains challenging [1-3]. The failure rate for new drugs targeting important CNS diseases is high compared to most other areas of drug discovery. Currently, the treatments available for CNS diseases are disappointing and primarily focus on relieving the symptoms rather than curing the disease. The main factors responsible for the failures in CNS drug development are a lack of understanding of the basic principles of CNS disease, the possibility of CNS side effects, and the inability of drugs to penetrate the blood-brain barrier (BBB).

The mechanisms underlying many CNS diseases have been understood for decades [3]. For example, Alzheimer’s disease (AD) is an age-related neurodegenerative disease and the most prevalent form of senile dementia in the world [4, 5]. Aβ peptides had been considered promising therapeutic targets for AD [6-8]. However, nowadays, the idea that AD is a multifactorial disease is growing in popularity, and many factors, including oxidative stress, neuroinflammation, energetic deficit, vascular damage, synaptic failure, axonal injury, tau pathology, and mitochondrial dysfunction, may contribute to AD onset and progression [9, 10]. Stroke is one of the most important causes of death and long-term disability in survivors [11]. Because many neuroprotective drugs validated in basic research have failed to show any effect against ischemic stroke in clinical settings, the concept of the neurovascular unit (NVU) has been proposed [12]. The NVU consists of endothelial cells, associated BBB tight junctions; basal lamina; pericytes; and parenchymal cells, including astrocytes, neurons, and interneurons [13]. The concept of NVU has not only changed and strengthened basic research into stroke but research into all CNS diseases [14, 15]. Drug development fails regardless of the progress of research into the mechanisms of CNS diseases; unfortunately, few drugs have been found to reproducibly improve outcomes in AD and stroke. AD drug development has proven to be unusually difficult, with a 99.6% failure from 2002 to 2012, and, currently, the success rate continues to be at the same low level [16]. Neuroprotective drugs for stroke have all failed in pivotal phase III efficacy trials [12, 17]. Brain metastasis is another challenging CNS disease. The metastasis of cancer to the CNS remains a devastating clinical reality, conveying an estimated survival time of less than one year, despite intensive treatment [18, 19]. Some primary tumors are more sensitive to therapies targeting specific molecular pathways, and a recent study found that these targeted therapies sometimes show activity in the brain and are delivered to the brain metastatic tumors [20, 21]. The medical needs of people suffering from brain metastases are still unmet, and much research and public attention are directed toward the treatment and prevention of primary cancer [22]. To date, there are no approved drugs for targeted brain metastasis.

One reason for the failure of clinical trials of CNS disease treatments is the CNS side effect, which is not experienced in animal studies; however, the main reason is the poor penetration efficacy across the BBB [3]. The BBB represents a bottleneck in CNS drug development and is the most important factor limiting the future growth of neurotherapeutics [23]. Essentially 100% of large-molecule pharmaceutics (>500 Da), including peptides, recombinant proteins, monoclonal antibodies, RNA interference (RNAi)-based drugs, and gene therapies and >98% of small-molecule drugs
do not cross the BBB [24]. Despite emerging demands for CNS drug development, in the 2010s, several high-profile global pharmaceutical companies decided to shut down major research projects into CNS drug development [25]. The major reason being that the development of new CNS drugs has become an increasingly high-risk activity with a poor success rate [25]. There is a clear need for the development of adequate models to further investigate the mechanisms of transport across the BBB in order to design better brain delivery strategies [26]. Drug development studies (including for the CNS) have historically used both in vivo and in vitro techniques. In vivo models directly utilize entire living animals, while in vitro models are constructions of artificial environments using cultured cells to mimic in vivo structures [27]. While in vivo models provide environments close to the human phenotype, they require extraordinary amounts of funding, time, and manpower per test, and their use is facing increasing pressure regarding the ethical issues involved [27, 28]. In vitro models can ameliorate these issues by offering the same environments in numerous arrays, as well as having less budget, time, and ethical constraints [27]. As described above, in vitro BBB models with high predictability are required for each stage of CNS drug development, such as candidate exploration, toxicity evaluation, and safety evaluation. Furthermore, most of the drugs already on the market have not been tested for their permeability into the CNS. An ideal in vitro model of the BBB would facilitate mechanistic studies of BBB tight junctions, transporters, enzymes, and macromolecular and immune cell trafficking and signaling [29]. It would also be need to be suitable for the rapid screening for the BBB permeability of both new CNS drug candidates and previously untested marketed drugs.

The attempts to develop new treatments for CNS diseases have costly and disappointing results and require a long period of time and high expenditure, whereas the repurposing of safe existing drugs provides a cost-effective and time-saving alternative [30, 31]. Drug repositioning, also known as drug repurposing, is based on the promiscuous properties of approved drugs, which encourages the re-examination of marketed drugs for new indications, and is an alternative approach to speeding up drug discovery that has become increasingly popular in recent years [31]. To date, the conventional de novo drug discovery process requires an average of around 13-15 years and 2-3 billion US dollars to approve and launch a drug [32, 33]. Drug repurposing usually focuses on drugs that have already cleared phase I safety trials but have yet to show efficacy for the intended indication. The preclinical study can often be bypassed, thereby reducing the overall cost of drug development. It is speculated that drug repurposing requires an average of 6.5 years and 300 million US dollars to approve and launch a drug [32, 33].

The process has been applied to find potential new uses for approved drugs or compounds for various CNS diseases, including dementia, neurodegenerative diseases, acute stroke, cancer, rare or orphan diseases, and metabolic disorders, and as antiviral, antibacterial, and analgesic drugs [31, 33-38]. In this review, the recent progress of in vitro BBB models have been introduced and drug repositioning for CNS diseases has been discussed [39].

2. BLOOD-BRAIN BARRIER

2.1. Structure and Function

The small capillaries of the brain are unique morphological and functional units that serve a variety of roles; they supply nervous tissue with oxygen and nutrients, such as glucose, amino acids, and neurotransmitter precursors, and are continuous nonfenestrated vessels with additional properties allowing them to tightly regulate the movement of molecules, ions, and cells between the blood and the CNS [29, 39]. This intensely restricting barrier capacity allows the BBB to firmly regulate CNS homeostasis, which is critical for proper neuronal function, as well as protect the CNS from toxins, pathogens, inflammation, injury, and disease [40-42]. Due to the absence of vascular fenestrations, nutrients, electrolytes, and metabolic waste are translocated across the endothelial cells via various transporters [43]. Glucose, amino acids, peptides, and proteins are actively transported down their concentration gradients from the vascular lumen to the brain parenchyma via carriers or receptors expressed at both the luminal and abluminal membranes of endothelial cells [41, 44-47]. Sodium pumps (Na⁺, K⁺-ATPase) in the abluminal membrane of the BBB regulate sodium influx into the brain in exchange for potassium [43, 47]. Potentially toxic substances, such as amylloide beta, a peptide implicated in the pathology of AD, are cleared from the brain parenchyma by receptors expressed at the abluminal membrane [41, 47]. Small lipophic molecules, with molecular weights <400 Da, can cross the BBB by passive diffusion through the lipid bilayer membranes of ECs, which is counteracted by enzymatic metabolism or active efflux from the vascular endothelium into the blood [41, 44-47]. Active efflux is normally accomplished by ATP-binding cassette transporters (ABC), such as P-glycoprotein (P-gp), breast cancer resistance proteins (BCRPs), and multidrug resistance-associated proteins (MRPs) [41, 46, 47]. ABC transporters are transmembrane proteins that use the hydrolysis of adenosine triphosphate (ATP) to drive the translocation of the substrate against its concentration gradient [48]. The capillaries of the brain form complex structures, consisting of several cell types; endothelial cells constitute the capillary wall and form the actual barrier and are surrounded by pericytes [29, 49]. The precise amount of the vascular surface that is covered by pericytes is still unclear but varies 22%-70% according to some reports (and is believed to be approximately 30%) [29, 50]. The endothelial cells and pericytes are surrounded by the basement membrane, and more than 99% of the capillaries are covered by astrocyte endfeet [50]. Both pericytes and astrocytes contribute to BBB properties and development and to the unique endothelial phenotype [51]. These roles are mediated by the expression and release of soluble factors, and they are possible through their anatomic proximity to ECs [52]. The pericytes and the astrocytes that interact with ECs enhance tight junctions (TJ) and reduce the gap junctional area, thus demonstrating that these cells have an important role in the restricted permeability and integrity of the BBB [53]. Brain capillary endothelial cells and the surrounding accompanying cells, including not only astrocytes and pericytes but microglia, neurons, mast cells, as well as circulating immune cells, constitute the NVU, a term encompassing the specialized and unique cellular structure and function of the brain microvasculature [42, 54].

With the accelerated pace of brain research in recent years and a growing understanding of the complexity of the brain and various brain-associated neurological diseases, the need for effective tools to enhance drug screening, diagnosis, and basic research is increasing [55]. Highly representative models of the CNS can play a critical role in meeting these needs [55]. Unfortunately, in vivo animal models lack controllability, are difficult to monitor and do not model human-specific brain-behavior accurately. While in silico computational models struggle to comprehensively capture the intertwined biological, chemical, electrical, and mechanical complexity of the brain. Thus, the appearance of high-quality in vitro BBB models is highly anticipated. In this review, in vitro BBB models for the development of CNS drugs have been focussed.

2.2. In Vitro BBB Models

In general, it is difficult to prepare an in vitro BBB model that perfectly mimics in vivo functions and properties. Several reports have indicated that tight junction function, which is one of the characteristics of the BBB, is reduced in vitro compared with in vivo. Additionally, it has been pointed out that the expression levels and polarity of transporters change [56-59]. Despite the disadvantages of the in vitro BBB model, cell culture-based BBB models are widely accepted as powerful tools for evaluating BBB physiology, pathophysiology, and pharmacology and drug development. The in vitro system can provide detailed mechanism elucidation using a simplified experimental system and facilitate multi-sample process-
To develop cell culture-based function by the co-culture of surrounding cells, such as pericytes and astrocytes, it is important to confirm that the function of endothelial cells is enhanced by the co-culture of surrounding cells, such as pericytes and astrocytes. In the case of the construction of BBB model using the insert membrane, brain endothelial cells are seeded onto the upper surface of the insert membrane while pericytes grow on the opposite surface of the membrane, and astrocytes are placed at the bottom of the well. In contrast to the static environment in standard culture conditions, blood vessels are exposed to shear stress in vivo. Based on this aspect, several flow-based BBB models, including the triple culture model, have been reported. To make a spheroid or organoid BBB model, mixed cells consist of endothelial cells, astrocytes, and pericytes are cultured in the same well and led to make 3D organized BBB models.

2.3. Validation for In Vitro BBB Models

To develop cell culture-based in vitro BBB models, we need to confirm that (1) BBB function can be quantitatively evaluated, (2) mature BBB characteristics are developed, and (3) the model mimics the in vivo situation.

To date, a variety of cell sources (rodent, porcine, bovine, and human) are available for the culture of in vitro BBB models. In recent years, the use of a flow model has been taken into consideration. Endothelial cells are the most important components of the BBB, and, to select appropriate endothelial cells, it is necessary to confirm the formation of a thin monolayer structure and to use markers of endothelial cells, such as von Willebrand’s factor, factor VIII, and PECAM-1 [60-64]. In addition to confirming the expression of typical tight junction proteins by mRNA and western blot, it is also important to confirm their localization in cell-cell borders by immunostaining [65-67]. Typical examples of objective evaluations of tight junction function include transendothelial electrical resistance (TEER) and permeability tests [56, 68, 69]. TEER is a widely accepted quantitative technique for measuring tight junction function in cell culture models of endothelial monolayers. TEER values are used to accurately assess the integrity of the cellular barriers before they are used to evaluate drug or chemical transport. The measurements can be performed in real-time without any cell damage and are generally based on the measurement of ohmic resistance or impedance across a wide spectrum of frequencies. The non-invasiveness of TEER measurements allow us to use the endothelial cells for other experiments, such as permeability tests, western blotting, and immunocytochemistry. The classical setup for measurement of TEER consists of a cellular monolayer cultured on a semipermeable filter insert, which defines the partition for apical (or upper) and basolateral (or lower) compartments (a Transwell system is normally used) [70]. For electrical measurements, two electrodes are separated by the cellular monolayer, i.e., one electrode is placed in the upper compartment and the other in the lower compartment. In theory, the ohmic resistance can be determined by applying a direct current (DC) voltage to the electrodes and measuring the resulting current [70]. The ohmic resistance is calculated, based on Ohm’s law, as the ratio of the voltage to the current [29]. However, DC currents can damage both the cells and the electrodes; therefore, to overcome this issue, an alternating current (AC) voltage signal with a square waveform is applied. In a widely used and commercially available TEER measurement system known as an Epithelial Voltohmmeter (EVOM), an AC square wave at a frequency of 12.5 Hz is used to avoid any charging effects on the electrodes and the cell layer [29, 70]. Caution is needed when comparing the TEER values among published reports because the values vary to some extent, not only because of differences in the actual junctional tightness but also differences in the measuring equipment (chopstick electrodes, cup electrodes, impedance measurements etc.), temperature, and handling of the cells during measurements [29, 70]. Furthermore, TEER is sometimes difficult to translate to real functional tightness, since the tightness of the endothelial monolayer depends on both the composition of tight junction complexes and the size of the compound of interest. To validate functional tightness, permeability tests with tracer molecules, such as Lucifer yellow (444 Da), sodium fluo-
rescein (376 Da), or sucrose (342 Da), are needed. TEER correlates with permeability for a given small hydrophilic molecule, but the correlation depends greatly on the size of the molecules and the experimental design [75-77].

3. VARIETY OF IN VITRO BBB MODELS

3.1. Parallel Artificial Membrane Permeability Assays (PAMPA)

The parallel artificial membrane permeability assay (PAMPA) is a procedure developed for a rapid determination of passive transport permeability that is widely accepted, especially in pharmaceutical research. In PAMPA, a filter plate coated with a liquid artificial membrane is used to separate two compartments: one containing a buffer solution of compounds to be tested (defined as donor compartment) and the other containing an initial fresh buffer solution (defined as acceptor compartment) (Fig. 2). Permeation determined with PAMPA using filters impregnated with a solution of phospholipids or hexadecane provided significant correlations with gastrointestinal absorption in humans [78, 79]. The development of in vitro assays for modeling BBB functionality and the high-throughput testing of drug candidates is a critical area of research. BBB-specific PAMPAs are widely used, non-cell-based tools for predicting the passive diffusion of compounds through the BBB in a relatively high-throughput manner [80, 81]. These models could predict the passive diffusion of the compounds. With the same experimental conditions, the porcine brain lipid was the most discriminating as the BBB model [79]. These assays are useful for eliminating the costs, animal-to-human translation inaccuracies, and ethical quandaries associated with in vivo animal testing [80]. Könczöl et al. have shown that the simple modification of PAMPAs is a potentially valuable strategy in the CNS drug discovery environment [82]. PAMPA has been extensively used for drug development, but it is beneficial to understand its limitations and to use it only to predict passive transport [83]. The false-positive values obtained in the compounds could be attributed to the compounds being substrates for active efflux [79]. Caution should be used when interpreting the results of the system because it lacks the complexity to accurately model active transport processes [80, 81, 83].

![Schematic diagram of PAMPA model.](image)

3.2. Cell Culture Devices and Extracellular Matrix to Construct In Vitro BBB Model

The discovery of cell culture inserts with porous filter membranes has allowed brain capillary endothelial cells to be used for permeability studies and the construction of co-culture in vitro models [84, 85]. So far, commercially available cell culture inserts have been applied most widely in the construction of in vitro BBB models. A researcher can choose from a variety of cell culture inserts with different pore diameters, pore densities, and membrane material, which affect the growth and function of endothelial cells, according to the research purpose. One benefit of using a culture insert is that it is possible to divide the space into two domains: the luminal (blood) and the abluminal (brain) side. Importantly, several reports have indicated that when culturing endothelial cells on large-pore-size membranes (more than a 3-μm pore size), the cells pass through the membrane and form a second layer on the undersurface of the insert membrane [86-88], and these in vitro models, which have a bi-endothelial layer, no longer represent the physiological BBB function. To avoid endothelial cells crossing the insert membrane, Vandenhaute et al. developed a new method involving the culture of endothelial cells on a dry-bottom insert (i.e., with no medium in the lower compartment) until a sufficient monolayer has developed. Additionally, properties of the insert membrane seem to affect the barrier function of the BBB model [88, 99]; therefore, selection of an appropriate insert membrane and optimization of culture conditions are critical in the model’s construction (Fig. 3).

The pore size and density of the membrane also affect the permeability of the substrate. Apparent permeability (Papp) is calculated using the following equation: Papp = (dQ/dt) / (S × C0), where dQ/dt is the cumulative amount of substrate in the receptor compartment versus time, S is the surface of the filter, and C0 is the initial concentration of the tracer in the luminal compartments. As the calculation of Papp includes the restriction effect of the insert membrane, Papp values tend to be low when using a small pore size and low pore density membrane. The influence of the membrane cannot be ignored, especially when examining the permeability of large molecules. In that case, the transendothelial permeability coefficient (P) can be used [90, 91]. Cleared volume is calculated from the concentration (C) of the tracer in the abluminal and luminal compartments and the volume (V) of the abluminal compartment by the following equation: Cleared volume = Cabluminal × Vabluminal / C_luminal. The average cleared volume is plotted vs. time, and the permeability × surface area product value for the endothelial monolayer (Pe) is calculated using the following formula: PS_endothelial = 1/Papp - 1/PSmembrane. Low pore density membranes generate the endothelial permeability coefficient. Although it is necessary to calculate the permeability of only the membrane using the transendothelial permeability coefficient, accurate permeability can be evaluated.

To promote cell attachment and growth on a culture device, several types of extracellular matrix (ECM) have been evaluated; collagen type I, type IV, and fibronectin are widely used in the construction of BBB models for their economy and other characteristics. BBB-related cells secrete several extracellular matrices: fibronectin, collagen IV, laminin, agrin, perlecain, SPARC, and nido-gen-1 [92-97]. These ECM play an important role in the maintenance and development of the BBB as a component of a NVU [98-100]. There are some reports of the effects of ECM on the function of the in vitro BBB model. Endogenous ECM mixtures derived from cultured endothelial cells, pericytes, and astrocytes strengthen the barrier properties, compared with endogenous ECM derived from non-brain endothelial cells (aorta) [101, 102]. In addition, collagen IV, fibronectin, and laminin have the ability to up-regulate the Papp function of porcine brain endothelial cells [103]. Katt et al. reported that iPS-cell-derived endothelial cells have a higher TEER value when the cells are cultured in cross-linked collagen I gel coated with fibronectin and collagen IV [104].

While the cell culture inserts are useful for culturing endothelial cells, it is difficult to expose the cells to fluid-induced shear stresses similar to those occurring in vivo blood vessels. Therefore, dynamic cell culture devices with micro-holes, such as three-dimensional tube structure and micro-fluidic devices, have been developed for the construction of BBB models [105-107].

3.3. Cells for Constructing In Vitro BBB Models

3.3.1. Immortalized Endothelial Cells

A large number of in vitro BBB models have been developed using immortalized and primary cultured endothelial cells. Immor-
A cultured model with C6 glioma cells has been used to develop an ECV304 cell line. ECV304 cells present some features of endothelial cells, and a co-culture of MDCK and Caco-2 cell lines, such as human bladder carcinoma (ECV304), Madin-Darby Canine Kidney (MDCK), and human colon carcinoma cell lines, including several transporters and junctional proteins. Among immortalized cell lines, some important BBB properties are retained; therefore, the cells have been used in various studies. The commercially available murine origin bEnd3 and bEND5 are widely used in BBB research, and the commercially available hCMEC/D3 cells, developed by Weksler et al., are well characterized by human brain endothelial cells. Reports indicate that hCMEC/D3 cells retain BBB properties, including several transporters and junctional proteins. Among immortalized cell lines, cEND and cerebEND, which are developed from mouse cerebral and cerebellar capillaries, respectively, possess well-retained barrier properties similar to primary cultured brain endothelial cells.

To predict a drug’s ability to penetrate the BBB, non-cerebral cell lines that are easy to handle and reproducible have been prepared from several species, such as mouse (bEnd3, bEND5, MBEC4, TM-BBB, cEND), rat (RBE4, GP8.3, GPNT, TR-BBB), bovine (TBMEC P11, tERT-TMEC), and human (hCMEC/D3, TY10, BB19, HBMEC/cilp) sources. Although leaky barriers are common disadvantages of these immortalized cells, some important BBB properties are retained; therefore, the cells have been used in various studies. The commercially available murine origin bEnd3 and bEND5 are widely used in BBB research, and the commercially available hCMEC/D3 cells, developed by Weksler et al., are well characterized by human brain endothelial cells. Reports indicate that hCMEC/D3 cells retain BBB properties, including several transporters and junctional proteins. Among immortalized cell lines, cEND and cerebEND, which are developed from mouse cerebral and cerebellar capillaries, respectively, possess well-retained barrier properties similar to primary cultured brain endothelial cells.

### Features of cell culture inserts

| Vender             | Corning, Falcon, Greiner, Millipore, Thermo Scientific Nunc |
|--------------------|---------------------------------------------------------------|
| Membrane material  | Polycarbonate, polyester, polyethylene terphthalate, hydrophilic PTFE |
| Well format        | 6, 12, 24, 96 well, 100 mm dish |
| Pore size          | 0.4, 1.0, 3.0, 8.0 μm |
| Pore density       | $1 \times 10^5$ ~ $1 \times 10^6$ pores/cm² |

(Decide on pore size, material and vender)

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### Features of cell culture inserts and calculation of permeability

Commercially available cell-culture inserts have been used most widely to construct in vitro BBB models. A researcher has to choose from cell culture inserts from different pore diameters, pore densities, and membrane material according to the research purpose. Bar in the representative image of Transwell indicates 5 μm. In general, the Permeability of the substrate is calculated by apparent permeability (Papp) or transendothelial permeability coefficient (Pe).

**Fig. (3).** Features of cell culture inserts and calculation of permeability. Commercially available cell-culture inserts have been used most widely to construct in vitro BBB models. A researcher has to choose from cell culture inserts from different pore diameters, pore densities, and membrane material according to the research purpose. Bar in the representative image of Transwell indicates 5 μm. In general, the Permeability of the substrate is calculated by apparent permeability (Papp) or transendothelial permeability coefficient (Pe).

### 3.3.2 Primary Cultured Endothelial Cells

While primary cultures are expensive, time-consuming, and require expertise, primary cultured cells have the advantage of retaining many in vivo properties. To construct an in vitro BBB model using primary cultured cells, there are several methodical considerations: the purification of endothelial cells, culture conditions, source of brain cells, and whether to co-culture with other BBB-related cells.

### 3.3.3 Purification of Brain Endothelial Cells

One method for obtaining brain capillary endothelial cells is based on the successful isolation of brain capillaries. Fractions of isolated brain capillaries contain several types of cells, such as endothelial cells, pericytes, and other brain-derived cells. Of the several attempts to eliminate contaminating cells, the development of a puromycin method has been successfully utilized to obtain pure endothelial cells. A puromycin method has been successfully utilized to obtain pure endothelial cells. This method is based on the fact that brain capillary endothelial cells express much higher amounts of P-glycoprotein than any other cells in the brain microvessel fractions, thus, they can tolerate toxic concentrations of P-glycoprotein ligand drugs while non-endothelial cells are eliminated. Puromycin is the best P-glycoprotein ligand to selectively kill contaminating cells. This selection appears to be more effective for capillary endothelial cells compared to large microvessels and could lead to tighter monolayers and better BBB models.

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**Table 1.** Features of cell culture inserts.

| Vendor | Corning, Falcon, Greiner, Millipore, Thermo Scientific Nunc |
|--------|---------------------------------------------------------------|
| Membrane material | Polycarbonate, polyester, polyethylene terphthalate, hydrophilic PTFE |
| Well format | 6, 12, 24, 96 well, 100 mm dish |
| Pore size | 0.4, 1.0, 3.0, 8.0 μm |
| Pore density | $1 \times 10^5$ ~ $1 \times 10^6$ pores/cm² |

(Decide on pore size, material and vender)
3.3.4. Culture Conditions

The culture conditions for in vitro BBB models are fundamental, as they can affect the properties of the BBB model. To appropriately maintain endothelial growth and function, several supplements are added to the culture medium. Glucocorticoid receptor agonists are widely used to improve the tightness of brain endothelial cells, whereas dexamethasone, corticosterone, and hydrocortisone can be used to strengthen barrier properties, increase TEER, and decrease Pe for paracellular markers [84]. Cyclic adenosine monophosphate (cAMP) is a prominent factor for inducing strong barrier properties in endothelial cells; cAMP increases barrier function through the upregulation of claudin-5 expression and phosphorylation of claudin-5 and myosin light chains [121, 122]. The elevation of cAMP using the cAMP analog, 8-(4-chlorophenylthio) cAMP (CPT-cAMP), a cell-permeable adenylate cyclase activator forskolin, and the phosphodiesterase (PDE) inhibitor are often applied to in vitro BBB models. However, for the examination of drugs, this model may influence intracellular cyclic nucleotides. Additionally, recent studies indicate that retinoic acid induces BBB properties in cultured brain endothelial cells and iPSC-derived endothelial cells [123, 124]. Of note, retinoic acid-treated human pluripotent stem cell-derived BMECs showed significantly induced tightness and a high TEER value (~ 5,000 Ω x cm²) [124].

3.4. Co-culturing with Other BBB-related Cells

Brain capillary endothelial cells are a major component of the NVU and dynamically interact with neighboring cells, including astrocytes, pericytes, perivascular microglia, and neurons. Cross talk between the cells of the NVU is crucial for the formation and maintenance of a functional BBB and homeostasis of the CNS [125]. Reconstructing the cross-talk between BBB-related cells using a co-culture system of porous filter membranes enhances the construction of an in vitro BBB model.

Astrocytes are well-characterized regulators of brain capillary endothelial-specific properties, including the induction of barrier function and the upregulation of enzymes and transporters [126, 127]. These findings support the use of a brain capillary endothelial cell/astrocyte co-culture as an in vitro reconstruction model, which has become the most widespread type of in vitro BBB model. The most commonly used source species for astrocytes are rodents, and they can be co-cultured with several species of endothelial cells from primary cultured and immortal cell lines. In contrast to astrocytes, pericytes are rarely used in BBB co-culture systems. Pericytes embedded in the brain capillary basement membrane are the nearest neighbors of endothelial cells, and they have a fundamental role in the stabilization of the brain capillary structure in vivo [101, 128]. Consistent with in vivo observations, recent reports indicate that pericytes, like astrocytes, play an important role in inducing BBB properties [74]. The barrier tightening effects of rodent and human pericytes have been demonstrated for rodent and human endothelial cells [73, 129]. However, there are conflicting data about the effects of pericytes on porcine endothelial cells. Several studies have reported that the pericyte-endothelial interaction increased matrix metalloproteinase activities and did not improve the barrier tightness of porcine brain endothelial cells (PBEC) [130, 131]. On the other hand, Thomsen et al. indicated that the co-culture of PBEC with rat pericytes resulted in significantly increased tightness [132]. Overall, the co-culture model using endothelial cells and pericytes is one of the most important tools in BBB research.

Considering the importance of astrocytes and pericytes in the induction of BBB properties, several groups have constructed triple-culture models [74, 132, 133]. Endothelial cells are grown in the presence of both astrocytes and pericytes, and a synergistic effect on the induction of BBB properties can be observed. To construct a BBB model using an insert membrane, brain endothelial cells are seeded onto the upper surface of the membrane, while pericytes grow on the opposite surface, and astrocytes are placed at the bottom of the well. In contrast to the static environment in standard culture conditions, blood vessels in vitro are exposed to shear stress. Because of this, several flow-based BBB models, including the triple-culture model, have been used [105-107]. Research groups have developed a spheroid/organoid type of BBB model by culturing a mix of endothelial cells, astrocytes, and pericytes in the same well, resulting in a 3D structure [133-135]. The spheroid BBB model is primarily suitable for drug screening tests.

Among the other types of brain cells, neural precursor cells (NPCs) and oligodendrocytes precursor cells (OPCs) also have the ability to induce BBB properties. During in vivo BBB development, brain capillaries invade the immature neural environment and interact with each other [136]. Consistent with this, differentiating embryonic NPCs, which contain neuron and glial cells, have been shown to be able to induce BBB properties in co-culture models [137, 138]. Recently, some reports indicated that OPCs play an important role in the development and maintenance of BBB properties [139-141]. In vivo studies have shown that platelet-derived growth factor receptor-α (PDGFR-α)-positive OPCs exist in the perivascular and parenchymal space, and OPC-specific TGF-β-knock-out mice show leaky barrier properties. Furthermore, the OPCs interacted with endothelial cells and pericytes in vitro. Conditioned medium from cultured OPCs decreased the endothelial permeability of FITC-dextran through the TGF-β signaling pathway and decreased pericyte proliferation. These data indicate that OPCs can induce BBB properties. As no studies have focused on the construction of an OPC-endothelial cell co-culture model, further research on the development and characterization of this type of BBB model are needed.

3.5. Source of Endothelial Cells

A variety of endothelial cell sources exist to construct the in vitro BBB model (Table 1). We here discuss some of them, especially focusing on primary endothelial cell culture.

3.5.1. Rodent Endothelial Cells

In vitro BBB models using brain endothelial cells derived from mice and rats are one of the most widely used. Mice and rats have a long history as experimental animals in various research fields; therefore, there is a wealth of accumulated knowledge and experimental tools that can be applied to BBB research. Several groups have designed rodent in vitro models, including monolayer, co-culture, and triple-culture designs. Compared to immortalized cells, primary cultured cells show a tight barrier function. Rodent endothelial cells generally display low to medium barrier function (TEER: 100-300 Ω x cm²), however, the barrier function is upregulated when endothelial cells are co-cultured with astrocytes and/or pericytes (TEER: over 500 Ω x cm²) [156]. Accumulated evidence indicates that rodent co-culture models enhance the expression of several transporters, receptors, and enzymes compared to monolayer models. Here we introduce two major methods for isolating rodent endothelial cells.

3.5.2. Isolation of Rat Endothelial Cells

Primary cultures of rat brain capillary endothelial cells (RBEC) are prepared from 3-week-old rats, as previously described [73, 74]. Meninges are carefully removed from the forebrains and gray matter minced into small pieces of approximately 1 mm³ in ice-cold Dulbecco’s modified Eagle's medium (DMEM), then dissociated by 25 up- and down-strokes with a 5-ml pipette in DMEM containing collagenase type 2 (1 mg/ml, Worthington Biochemical Corp., NJ, USA), 300 μl DNase (15 μg/ml), and gentamycin (50 μg/ml) and digested in a shaker for 1.5 h at 37 °C. The cell pellet is separated by centrifugation in 20% bovine serum albumin (BSA)-DMEM (1000 x g, 20 min). The microvessels obtained in the pellet are further digested with collagenase-dispase (1 mg/ml, Roche Applied Sciences, Basel, Switzerland) and DNase (6.7 μg/ml) in
Table 1. Different cells to construct *in vitro* blood-brain barrier model.

| - | Cells | Features | References |
|---|---|---|---|
| Non-brain derived cells | ECV304 | Human bladder cancer derived epithelial cell line. Exhibit both endothelial and epithelial characteristics. | [113, 142] |
|  | Caco-2 | Human colon carcinoma cell line. High barrier properties. | [115, 143] |
|  | MDCK | Madin-Darby Canine Kidneys High barrier properties. | [115, 117] |
|  | Peripheral endothelium | HUVECs are widely used for endothelial biology. Co-cultured with astrocytes. | [144, 145] |
| Brain-derived cells | RBE4, GP8.3 TR-BBB | Rat brain-derived cell lines. Low barrier properties. | [109, 146] |
|  | bEnd.3, MBEC4 TM-BBB, cEND | Mouse brain-derived cell lines. Low barrier properties. cEND possess well-retained barrier properties. | [109, 147, 148] |
|  | hCMEC/D3 | Mouse brain-derived cell lines. Low barrier properties. One of the well-characterized cell line. | [111, 149] |
|  | Mouse, Rat | Low yield of endothelial cells from one brain. Possible to comparison with *in vivo* data. | [73, 150] |
|  | Bovine, porcine | High yield of endothelial cells from one brain. High barrier properties. | [151, 152] |
|  | Monkey | High yield of endothelial cells from one brain. Primates model. | [153, 154] |
|  | Human | Human data. Difficult to obtain normal tissue stably. | [155] |
| Primary cultured cells | Artificial membrane (IAM, PAMPA) | High throughput assay. Predict for passive transcellular transport. | [79] |
|  | iPS-derived cells | Several groups developed *in vitro* BBB model using iPS-derived cells. | [136] |

DMEM for 1 h at 37°C. Microvessel endothelial cell clusters are separated on a 33% continuous Percoll (Pharmacia, Uppsala, Sweden) gradient, collected, and washed twice in DMEM before plating on 35 mm plastic dishes coated with collagen type IV and fibronectin (both 0.1 mg/ml). RBEC cultures are maintained in DMEM/F12 supplemented with 10% plasma-derived serum (PDS, Animal Technologies Inc., MD, USA), basic fibroblast growth factor (bFGF, Roche, Applied Sciences, Basel, Switzerland, 1.5 ng/mL), heparin (100 μg/ml), insulin (5 μg/ml), transferrin (5 μg/ml), sodium selenite (5 ng/ml) (insulin-transferrin-sodium selenite media supplement), gentamycin (50 μg/ml) and puromycin (4 μg/ml) (RBEC medium I) at 37°C with a humidified atmosphere of 5% CO₂/95% air, for 2 days [119]. On the third day, the cells receive a new medium containing all the components of RBEC medium I except puromycin (RBEC medium II). When the cultures reached 80% confluency (4th day in *vitro*), the purified endothelial cells are passaged by a brief treatment with trypsin (0.05%, w/v)-EDTA (0.02%, w/v) solution, and used to construct various types of *in vitro* BBB models.

A major advantage of using the rat BBB model is that syngeneic co-cultures can be established, and the results obtained from
rat BCECs can be correlated with in vivo data from the same species, and even strain, of the rat. In addition, the genome and transcriptome of rats are well understood, and a large set of antibodies are available for rat antigens.

3.5.3. Isolation of Mouse Endothelial Cells

The following describes the preparation of primary mouse BECs, according to Coisne et al. [157]. Briefly, meninges are carefully removed from forebrains and gray matter minced into small pieces. Preparations are pooled and ground with a Dounce homogenizer in Dulbecco’s Modified Eagle’s Medium/Nutrient Mixture F-12 Ham (DMEM/F12; Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with gentamicin (50 µg/ml, Sigma-Aldrich Co., St. Louis, MO, USA). The resulting homogenate is mixed with 30% dextran (v/v, molecular weight 100,000–200,000, Sigma-Aldrich Co., St. Louis, MO, USA) in DMEM/F12 supplemented with 0.1% BSA; Sigma-Aldrich Co., St. Louis, MO, USA). The suspension is centrifuged at 3000 × g for 25 min at 4 °C. The pellet is suspended in DMEM/F12 and the supernatant centrifuged again under the same conditions. After the second centrifugation, the supernatant is discarded and the pellet resuspended in DMEM/F12. Then the pellets are filtered through a 70 µm nylon mesh and digested in collagenase/dispase (2 mg/ml, Roche Applied Science, USA) and DNase I (10 µg/ml, Sigma-Aldrich Co., St. Louis, MO, USA) for 30 min. The digested solution is filtered through a 20 µm nylon mesh and seeded on collagen type IV/fibronectin-coated dishes (both from Sigma-Aldrich Co., St. Louis, MO, USA). Cultures are maintained in medium composed of DMEM/F12 supplemented with 10% PDS (Animal Technologies, Inc., USA), 1% GlutaMAX ( Gibco), basic fibroblast growth factor (bFGF; Roche Applied Sciences), heparin, insulin, transferrin and sodium selenite supplement, and puromycin (4 µg/ml, Sigma-Aldrich Co., St. Louis, MO, USA) [119]. Twenty-four hours after plating, red blood cells, cell debris, and nonadherent cells are removed by washing with a medium. On the third day, the puromycin is removed from the medium. When the cultures reached 80% confluency (5th day in vitro), the purified endothelial cells are passaged by brief treatment with 0.25% Trypsin-EDTA (Invitrogen, Life Technologies) and used to construct in vitro BBB models.

The mouse is widely used as an animal model in biomedical research. This is not surprising as 99% of mouse genes have human gene counterparts, and its physiology and genetics have been studied extensively and can be easily compared with humans. Technologies, such as transgenics, have been developed over decades for studying mouse genetics and the function of specific genes. Many human disease model mice have been developed to advance the study of disease pathogenesis and to evaluate the efficacy and toxicity of various candidate drugs.

3.5.4. Porcine and Bovine BBB Models

Porcine and bovine brains are commonly used for the isolation of brain capillaries, owing to their brain size, economic advantages, and availability. Obtaining cells from laboratory animals is very expensive and requires large amounts of animal sacrifice. Primary cultures of porcine and bovine endothelial cells have been widely reported to maintain many important barrier characteristics and transport pathways [84, 151, 158, 159]. Therefore, it has been suggested that porcine and bovine in vitro BBB models are effective tools for researching BBB and in pharmaceutical permeability screening. A unique property of PBEC is that a physiological concentration of hydrocortisone considerably improves barrier properties in serum-free culture conditions [151]. Additionally, Thomsen et al. reported that a triple-cultured model using porcine endothelial cells, pericytes, and rodent astrocytes enhanced barrier function and the expression of claudin-5, occludin, and BCRP [132]. The astrocytes are often derived from rat pups, as they grow faster than astrocytes obtained from older animals. Thomsen et al. successfully created a triple-cultured model using cells isolated from 6-month-old domestic pig brains, and several groups have developed an in vitro BBB model using primary cultured bovine brain endothelial cells [132]. A major advantage of bovine brain endothelial cells is the high yield of endothelial cells per brain, thanks to the cells’ tolerance of several rounds of subculture [158]. Generally, BBB properties, especially barrier function, are rapidly lost during the subculture of rodent brain endothelial cells. Bovine endothelial cells have a strong barrier function and retain these properties even after subculture. The co-culture model of bovine endothelial cells and rat astrocytes is widely used in signaling and transport studies, as well as in the characterization of fundamental BBB properties [160].

3.5.5. Human and Monkey In Vitro BBB Model

Although the brain tissues of various species are used to obtain capillary endothelial cells, the human brain is also an important source. However, it is difficult to use normal human brain tissue because of ethics and constraints in obtaining the samples. In addition, when the brain sample contains pathological tissue, the endothelial cells isolated from the tissue may have a different property to normal human endothelial cells. Hence, to construct a human in vitro BBB model, commercially available brain endothelial or immortalized cells are mainly used. Furthermore, several groups have developed a human BBB model using iPS cell-derived BBB-related cells [136].

Some studies have used a commercially available monkey BBB model, constructed from monkey brain endothelial cells, rat pericytes, and astrocytes [154, 160, 161]. The monkey model displays a high TEER value (more than 800 Ω × cm²), expresses tight junction proteins (ZO-1, occludin, claudin-5) at the cell-cell junction, and can be applied to transport assays for chemical compounds and physiological peptides.

3.6. Application of In Vitro BBB Models (Fig. 4)

3.6.1. Using In Vitro BBB Models to Evaluate BBB Physiology

Endothelial cells of the blood vessels of different organs possess different characteristics and are regulated by specific local signals [162, 163]. Brain capillaries have a special function as the anatomical basis of the BBB, which acts to maintain brain homeostasis and has evolved to protect the health and activity of fragile neurons. The brain environment is maintained through the selective uptake and restriction of substances entering the CNS from the blood, which is achieved through interactions among components of the NVU: brain capillary endothelial cells, astrocytes, pericytes, perivascular microglia, and neurons. In addition to crosstalk along the NVU, substances in the blood fluid also affect the functions of the BBB. In vitro BBB models have provided numerous insights into these complex mechanisms behind the development and maintenance of the BBB.

3.6.2. In Vitro BBB Models and Evaluating BBB Pathophysiology

In vitro BBB models can be used to elucidate the role of BBB in disease states. Because the BBB plays a critical role in the protection of the brain against harmful substances in the peripheral fluids, disruption of the BBB evokes brain edema and allows the penetration of toxic substances and inflammatory cells, leading to neuronal damage. Accumulating evidence suggests that disruptions to the adhesion between brain capillary endothelial cells play a role in the onset and progression of several CNS disorders [42, 98]. In vitro BBB models are powerful tools to evaluate the mechanisms of BBB disruption under these pathophysiological conditions and in the search for BBB protective drugs to prevent the development of CNS diseases.

3.6.3. Using In Vitro BBB Models in Drug Development

The BBB protects the brain from harmful blood-borne substances. On the other hand, it is a major barrier to be overcome when developing CNS drugs, as it is the main regulator of drug
transport into the CNS. Several potential routes for permeation across the BBB are known, such as passive diffusion, ABC transporter efflux, carrier-mediated influx, receptor-mediated transcytosis, and adsorptive-mediated transcytosis. The prediction of effective drug penetration across the BBB using an in vitro BBB model is important for the development of centrally acting drugs.

Another method to predict BBB permeability is using in vitro BBB models. Several reports indicated that immortalized cells less able to predict BBB permeability than primary cultured cells [165]. Although in vitro models using primary cultured cells are expensive and time-consuming compared with that of cell lines, primary cultured cells have the advantage of retaining many in vivo properties. In fact, some in vitro co-cultured BBB models have a good correlation to in vivo BBB permeability [74, 152]. Though in vitro BBB model using primary cultured cells is low to medium throughput, the prediction of effective drug penetration across the BBB using an in vitro BBB model is important for the development of centrally acting drugs [26]. Species differences, especially between humans and other species, have important implications for the drug discovery and development process. Using a quantitative targeted absolute proteomics method, Ohtsuki et al. showed that P-glycoprotein is expressed less abundantly in monkey and human brain capillaries than in mouse brain capillaries [166]. In addition, a PET study demonstrated that the brain penetration of an MDR1 substrate, such as $^{13}$C GR205171, was greater in humans and monkeys than in rodents [167]. Considering these data, the triple co-culture model using monkey endothelial cells is a practical primate in vitro BBB model for basic research and drug permeability assays.

4. DRUG REPOSITIONING

Decades of effort have gone into making in vitro BBB models, and the qualities of the models are improving, as mentioned previously, but the development of CNS drugs is still a major challenge [29]. The attempts to develop new treatments for CNS diseases too frequently meet costly and disappointing results and they require
both long timespans and high expenditure [31]. However, the repurposing of safe existing drugs to new indications provides a cost-effective and time-saving alternative [30, 31]. Drug repositioning or repurposing uses the versatile properties of approved drugs to reassign then to a new purpose. This alternative approach using drug discovery fast-tracking is becoming increasingly popular [30]. Drug repositioning is particularly well-suited to the public sector, where off-patent agents can undergo high-throughput in vitro screening for their ability to interact with identified molecular targets [35]. The term drug repositioning is frequently used in the literature has several synonyms such as drug repurposing, drug reprofiling, which have been interchangeable. To date, no common consensus exists the definition of drug repositioning or similar terms. Drug repositioning is referred to the drugs approved for one disease which are used as a structural template for synthesis of derivatives active against another disease, while drug repurposing is referred to the old drugs that can be used without modification for new uses [168].

Historically, drug repositioning has been a largely unintentional, serendipitous process that takes place when a drug is found to have an off-target effect or a previously unrecognized on-target effect that could be applied to another purpose. Perhaps the most famous example of successful repositioning effort is sildenafil. Sildenafil, originally developed as a angina pectoris, has been repurposed for the treatment of erectile dysfunction and, subsequently, pulmonary arterial hypertension. Another famous example is thalidomide, originally developed as a sleep-inducing drug but discontinued due to fetal teratogenicity, which is now used against multiple myeloma because of its anti-angiogenic effects [169]. Drug repositioning has the potential to facilitate drug discovery by physicians and pharmacists during daily clinical practice. In addition, drug repositioning also includes pharmaceuticals that are confirmed to be safe with clinical trials, but the effectiveness cannot be proven and their development is discontinued. The method of searching for new applications for marketed drugs that have been discontinued is called drug rescue; although, it may be classified as drug repurposing when their clinical application to other diseases is predicted using the known drug effects and side effects. In recent years, drug repurposing by drug reprofiling is becoming mainstream, and it is used to comprehensively analyze the actions of existing drugs at the molecular level using the latest analysis methods, and to examine the possibility of its use as a therapeutic drug for other diseases [170]. In vitro systems, such as high-throughput screening, are often used for effective discovery, and in silico systems, based on drug and disease databases, can be used for evaluation. Battah et al. recently reported an integrated screening protocol combining in silico and in vitro approaches to uncover the antimycobacterial potential of existing drugs. Their in silico system provided a series of marketed drugs possessing significant antitubercular activity levels, as assessed by different in vitro system [168].

Drug repositioning is, simultaneously, a very old and very new method. In fact, the number of papers related to drug repositioning has been increasing in recent years. In PubMed, since 2013, more than 2300 publications have been indexed based on the keywords drug repositioning. More than 2300 publications have been indexed based on the keywords drug repositioning since 2013 and that number is increasing year on year (Fig. 5).

The CNS is an important area for drug repositioning, and a well-known example of this is amantadine [171]. Amantadine was first recognized as an antiviral compound in the 1960s and was approved by the FDA for influenza prophylaxes in 1966. It was then repositioned for Parkinson’s disease, based on a case study in 1968 [172, 173]. The mechanisms are not fully elucidated, and, subsequently, a huge variety of indications of amantadine have been explored, from fatigue in multiple sclerosis, enuresis nocturna, ADHD, to pathological gambling and recovery after a head injury [172, 173]. Amantadine is currently used as an extended-release formulation for levodopa-induced dyskinesia in Parkinson’s disease.

The underlying pathophysiology of CNS diseases are continuously being unraveled; therefore, it is beneficial to revisit the receptor profiling and mode of action of marketed drugs. However, most of the diseases within the CNS, especially psychiatric or neurological diseases, are still poorly understood in terms of their underlying pathophysiology and biological mechanisms [174]. To date, the exact mode of action by which many of the currently approved CNS drugs exert their effect is still not fully understood. Thus, the current disease characterizations within CNS are mainly based on clinical aspects, rather than the underlying pathophysiology [174]. It is not surprising that, despite the high prevalence of CNS disorders in the overall population and the high demand in this area, the discovery and development of drugs for CNS diseases has one of the lowest success rates. Therefore, drug repositioning for CNS diseases is becoming increasingly popular [175]. Successful drug repositioning examples in the CNS area are listed in Table 2.

| Drug Name | New Indication                        | Original Indication |
|-----------|---------------------------------------|---------------------|
| Thalidomide | Multiple myeloma                       | Sleep inducer       |
| Amanantadine | Parkinson’s disease                   | Influenza           |
| Valproic acid | Migraine                              | Epilepsy            |
| Zonisamide | Parkinson’s disease                   | Epilepsy            |
| Dabrafenib | Parkinson’s disease                   | Melanoma            |
| Edaravone | Amyotrophic lateral sclerosis         | Stroke              |
| Topiramate | Obesity                               | Epilepsy            |

Since CNS drugs already on the market have been confirmed as possessing some effects on the CNS, it is entirely conceivable that they could act on other CNS diseases. In addition, the fact that the problem of side effects has already been overcome is also an advantage in terms of drug development. Caban et al. recently reported that the majority of CNS drugs successfully approved as a result of drug repositioning were originally for other CNS disorders [174]. On the other hand, the CNS drugs still in development presented a lower proportion of CNS drugs. Further research will be needed to validate their results, but one reason for this may be the problem of BBB penetration. Most drugs and drug candidates cannot reach the brain; however, already approved CNS drugs have a greater potential to pass through the BBB, which is the highest obstacle to the
development of CNS drugs. Indeed, approximately 30% of CNS drugs have been repurposed two or more times as other CNS therapeutics [174]. In vitro BBB models can be powerful tools to validate the candidates of drug repositioning (Fig. 6A & B). In this perspective, we provide some representative CNS diseases that the focus of drug repositioning projects.

4.1. Alzheimer’s Disease

The underlying pathology of AD is complex and includes, amyloid beta (Aβ) plaque depositions, neurofibrillary tangles, brain atrophy and neurodegeneration, neuroinflammation, impaired neurogenesis, and vascular and BBB disruptions [176-183]. Despite recent advances in AD research, pathophysiological characterization remains incomplete, which hampers the development of effective treatments [184]. In fact, currently, there are no effective pharmacological treatments for AD [185]. In this situation, adopting a different strategy based on the repurposing of drugs that are approved for other disorders is attractive [186]. Kumar et al. studied the molecular interactions of already known antipsychotic drugs with the various protein targets implicated in AD using in silico studies [185]. They screened approximately 150 antipsychotic drugs and performed molecular docking on five major protein targets: acetylcholinesterase (AchE), butyrylcholinesterase (BuChE), beta secretase cleavage enzyme 1 (BACE 1), monoamine oxidase (MAO), and N-methyl-d-aspartate (NMDA). Their study has highlighted the potential of using leading antipsychotic drugs, such as pimozide, bromperidol, melperone, anisoperidone, benperidol, and anisopirol, against multiple targets associated with AD. In their study, benperidol was found to be the best candidate for the cholinergic (AchE and BuChE), monoaminergic (MAO A), and glutamatergic (NMDA) systems and beta secretase cleavage enzyme (BACE 1) [185]. Approximately 40% of clinical trials for AD currently in progress worldwide are using drug repositioning [6]. In these clinical trials, other original CNS drugs, such as rasagiline, levetiracetam, and riluzole, are being applied to AD, and there have been reports of other candidates, such as insulin, pioglitazone, leukotriene, and candesartan [6, 187-189]. This is likely to be a response to the recent announcement that AD is a complex multifactorial disorder, not dominated by one dominant biological factor, such as beta-amyloid, and including many relevant pathologic factors. Drug repositioning approaches may lead to a paradigm shift in the treatment of AD patients.

4.2. Metastatic Brain Tumor/Glioma

As there is very little research on metastatic brain tumors, there are few reports on drug repositioning studies, and they’re only a few reports on breast cancer drug repositioning. The financial and time constraints of anticancer drug development are becoming increasingly greater barriers. It is becoming increasingly challenging to discover new anticancer drugs; therefore, the success rate is declining [190]. In the field of oncology, there are many examples of repurposing successes, proving the potential of this strategy. Aspirin, which is originally an antipyretic drug, was repurposed to treat colorectal cancer, and metformin, which is an anti-diabetes drug, was repurposed to treat breast cancer, prostate cancer, etc. [191, 192]. Previous epidemiologic studies reported that there are few patients with psychosis who develop cancers. In a study comparing schizophrenic patients with no history of cancer with a control group during a nine-year follow-up, the cancer incidence was 1.93% in the schizophrenic patient group, compared with 2.97% in the control group [193]. This result suggests that the likelihood of developing cancer among patients with schizophrenia was less than among the non-schizophrenic group. Other studies have shown an overall decreased cancer incidence in schizophrenic patients using neuroleptic drugs, implying that these drugs may have anticancer potential [194, 195]. In addition, some anti-schizophrenia drugs, such as trifluoperazine and chlorpromazine, have shown anticancer efficacies in preclinical studies [196, 197]. Fluphenazine hydrochloride is another commonly prescribed antipsychotic drug, and a few studies have reported its efficacy in the treatment of breast cancer [198]. As an anti-schizophrenia drug, Fluphenazine hydrochloride can penetrate the BBB to reach a relatively high concentration in the brain. Xu et al. evaluated the activity of fluphenazine hydrochloride in the treatment of triple negative breast cancer (TNBC) and brain metastases, and fluphenazine hydrochloride exhibited good anti-metastatic potential in a mouse brain metastasis model with an inhibition rate of 85% [199]. The drug also showed a strong inhibitory effect on spontaneous lung metastasis. Moreover, fluphenazine hydrochloride did not cause serious side effects in the mice. Their results prompted further preclinical and clinical investi-
gations into repurposing fluphenazine hydrochloride for treating metastatic TNBC patients, who urgently need new treatment options. The exact mechanisms of fluphenazine hydrochloride’s anti-cancer effects are still unknown. Previous studies have shown that the various mechanisms of antipsychotic agents may be used to fight cancer, including the induction of autophagy, dysregulation of cholesterol homeostasis, and disruption of lysosomal homeostasis [196, 197, 200]. This suggests that some antipsychotic drugs can prevent the development of cancer, and further investigation is necessary.

4.3. Stroke
A common misperception of neuroprotection research is that, although many treatments work in animals, nothing works in people. This perception has been repeatedly reinforced by reports of unsuccessful or mixed outcomes in trials of candidate neuroprotec-
tants in acute stroke patients [201, 202]. If animal experiments are indeed unable to inform clinical decision-making, then serious doubts are raised about the utility of animal models of stroke and about the ethics of continuing animal experiment practices. The differences among species may play a role in the fact that of 1,026 treatments tested on animal models for stroke therapy, nothing has been effective in clinical trials [202]. In this situation, drug repositioning is a realistic option. During a stroke, the occlusion of a cerebral vessel leads to a rapidly progressing cascade of events and to the destruction of critical brain tissue. This includes not only neurons but also other cells, including glial and vascular cell types and the intervening matrix [203]. Promising targets have been identified for vascular protection after strokes, such as the inhibition of endogenous mediators of vascular damage (superoxide, endothelin, matrix metalloproteases, cytokines, and caspases) and the stimulation of endogenous protectors (nitric oxide, angiopoietin-1, vascular endothelial growth factor, and superoxide dismutases) [203]. Several of these targets can be approached with repurposed drugs, including statins, angiotensin II receptor blockers, minocycline, and growth factors such as erythropoietin [204]. Other vascular protection strategies that have been successful in experimental models of cerebral ischemia are lowering of blood pressure [205, 206], melatonin [207], minocycline [208, 209], statins [210, 211], and fasudil, a Rho-kinase inhibitor [212].

Vascular protection seems to be a promising strategy for improving stroke outcome, as vascular function is critical to both cardiovascular diseases and ischemic cerebrovascular diseases [31]. Vascular-function-related biological processes and pathways may be the key links between cardiovascular diseases and ischemic cerebrovascular diseases. Zhao et al. reported that a multi-database, in silico target identification, gene function enrichment, and network pharmacology analysis integration method was applied to investigate the 119 FDA-approved cardiovascular disease drugs for ischemic cerebrovascular diseases repurposing. As a multi-target pleiotropic drug, carvedilol was investigated for its potential as an ischemic cerebrovascular disease therapy. Carvedilol is a nonselective $\beta$-adrenergic blocking agent with $\alpha_1$-blocking activity, and it shows pleiotropism by affecting 17 targets. Their results indicated that the mode of action of carvedilol for ischemic cerebrovascular disease treatment may be closely linked to vascular function regulation, and the mechanism is multi-target and multi-signaling pathway-related [31]. In the future, this method of computational drug discovery and drug repositioning based on the biomolecular profile is expected to increase.

Attempts to develop new drugs for acute ischemic stroke are still struggling; however, acute stroke therapy has now entered a new era of highly effective reperfusion through the development of the stent retriever and vacuum aspiration devices that can directly remove the thrombus from inside the occluded cerebral artery directly [12]. The recent publication of five positive thrombectomy trials in 2015 and subsequent publications, including the American Heart Association/American Stroke Association guidelines, support the evidence for the effects of mechanical thrombectomy [213-219]. Even in the era of rapid and effective recanalization using endovascular approaches, the percentage of patients with good outcomes varies between 33 and 71%. Furthermore, these trials used various forms of pre-thrombectomy imaging criteria and/or time limitations to exclude patients with significant infarcted cores. Thus, while clinical outcomes continue to lag behind the high rates of technical success, there may be an opportunity to use adjuvant neuroprotec-
tants to extend the window for intervention or to reverse damage that is currently viewed as unsalvageable based on radiographic imaging alone, thereby increasing the number of patients eligible for thrombectomy [220]. Considering these facts, combining neuro-protection with intravenous or intra-arterial reperfusion therapy is now an important next step in the development of acute stroke therapies. Mechanical thrombectomy is now at the forefront of the treatment of large-vessel acute ischemic stroke [221]. Selective intra-arterial access has opened a new avenue for neuroprotection in acute ischemic stroke that has the potential to maximize the local benefits while minimizing systemic effects [220, 221].

In animal models of acute ischemic stroke, verapamil, nitroglycerin, erythropoietin, and platelet-rich plasma have been evaluated via the intra-arterial route [220, 222, 223]. Among them, one possible candidate neuroprotectant for stroke therapy, verapamil, a calcium channel blocker, was an effective intra-arterial adjunct in a preclinical mouse focal ischemia model [220, 221]. Fraser et al. recently reported on an in vivo intra-arterial dose-response evaluation of their mouse stroke model. Furthermore, they have already conducted a human Phase I trial, Superselective Administration of Verapamil During Recanalization in Acute Ischemic Stroke (SAVER-I), to evaluate the feasibility and safety of the drug’s administration in the human disease. The SAVER-I clinical trial showed no evidence that IA verapamil increased the risk of intracranial hemorrhage or other adverse effects/procedural complications in human subjects [220]. Since mechanical thrombectomy is now the gold standard for acute ischemic stroke treatment, neuroprotective strategies via the intra-arterial route are highly anticipated.

4.4. Parkinson’s Disease
The exact pathophysiological mechanisms underlying neurodegeneration in Parkinson’s disease (PD) are not well understood. De novo drug discovery and development in PD is time-consuming, expensive, and risky process same as other CNS diseases. Therefore, it is reasonable that pharmaceutical companies are facing a paradigm shift in how drugs for PD are discovered and developed. To overcome the high attrition in drug development, pharmaceuti
cal companies are increasingly exploring drug repositioning. In fact, amantadine, originally developed in the 1960s as a prophylactic against several forms of influenza, has approved for the treat-
ment for PD. This repositioning from an anti-flu compound to an anti-Parkinson drug was initiated by a case observation in 1968 of a 58-year-old woman with moderately severe Parkinson’s disease. This patient told the treating neurologist that while talking aman
tadine hydrochloride 100 mg tablets to prevent the flu, she experi
cenced a remarkable remission in her symptoms of rigidity, tremor, and akinesis [172]. Although the compound is hydrophilic it easily penetrates the blood-brain barrier, due to active transport probably via a proton-coupled organic cation antiporter [224]. Interestingly, there have been facts emerging, amantadine has a great variety of action in other indications such as multiple sclerosis, traumatic brain injury, cancer pain. The mechanisms of action of amantadine related to the various indications will be totally different among the indications. Zonisamide is another representative drug repositioning example. It has been developed as an anti-epileptic drug in the 1980s. Murata et al. used zonisamide to treat epilepsy in a patient with PD and serendipitously found that not only epilepsy but symp-
toms related to PD were improved [225]. The anti-epilepsy effect of
zonisamide has been related to the inhibition of voltage-dependent sodium channels and T-type calcium channels. While the anti-PD effect of zonisamide may be related to the inhibition of dopamine metabolism due to inhibition of monoamine oxidase-B, the stimulation of dopamine release from striatum, and the blockade of T-type calcium channels. Based on these data and subsequent clinical trials, zonisamide was approved in Japan as an anti-PD drug in 2009.

Currently available treatment for PD primarily focus on stimulation of dopaminergic signaling and can provide symptomatic relief for a limited time but little effect on nonmotor symptoms. And none of the drugs have shown to affect the progressive pathological and clinical decline. As with other multifactorial genetic disorders, genome-wide association studies (GWASs) found multiple risk loci for PD, although their clinical significance remains uncertain. Uenaka et al. report the identification of candidate drugs for PD by a method using GWAS data and in silico databases [226]. They identified 57 Food and Drug Administration-approved drug families as candidate neuroprotective drugs for PD. Among them, dabrafenib, which is known as a B-Raf kinase inhibitor and is approved for the treatment of malignant melanoma, showed remarkable cytoprotective effects. Their data indicated that dabrafenib exerts protective effects against neurotoxicity associated with PD. They also confirmed the effectiveness of this in silico screening method by using animal model. Drug repurposing is a promising strategy for drug development because the safety data of these drugs in human patients are already available [227]. However, it is inefficient to examine all FDA-approved drugs, because of their vast number. In silico drug screening, which links the data of GWAS to drug/protein-protein interaction databases, may narrow down candidate drugs for many polygenic diseases and save cost and time.

CONCLUSION

In vitro BBB models can be essential tools for basic research and pharmaceutical screening and drug repositioning by mimicking clinical settings.

Drug repositioning can minimize the cost and time span of CNS drug development. To date, two-thirds of repositioned drugs are the result of a process of screening through careful observation by the treating physicians. In addition, pharmacists, and other medical staff, as well as basic researchers, should pay attention to the daily observations of patients, the appropriate conduct of clinical trials, and the drug’s un-anticipated effects on patients. Furthermore, in silico drug repositioning, which involves network analysis, data mining, and machine learning, is also expected to play an important role in future treatment developments.

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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