Human iPSC-Derived Blood-Brain Barrier Models: Valuable Tools for Preclinical Drug Discovery and Development?

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Translating basic biological knowledge into applications remains a key issue for effectively tackling neurodegenerative, neuroinflammatory, or neuroendocrine disorders. Efficient delivery of therapeutics across the neuroprotective blood-brain barrier (BBB) still poses a demanding challenge for drug development targeting central nervous system diseases. Validated *in vitro* models of the BBB could facilitate effective testing of drug candidates targeting the brain early in the drug discovery process during lead generation. We here review the potential of mono- or (isogenic) co-culture BBB models based on brain capillary endothelial cells (BCECs) derived from human-induced pluripotent stem cells (hiPSCs), and compare them to several available BBB *in vitro* models from primary human or non-human cells and to rodent *in vivo* models, as well as to classical and widely used barrier models [Caco-2, parallel artificial membrane permeability assay (PAMPA)]. In particular, we are discussing the features and predictivity of these models and how hiPSC-derived BBB models could impact future discovery and development of novel CNS-targeting therapeutics. © 2020 The Authors.

Keywords: blood-brain barrier (BBB) *in vitro* model • CNS disease • drug permeability screening • human-induced pluripotent stem cells (hiPSC) • preclinical drug discovery

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INTRODUCTION

Effectively targeting diseases of the central nervous system (CNS) remains an unmet clinical need. Efficient delivery of therapeutics across the blood-brain barrier (BBB) poses a demanding challenge for CNS drug development and translation of basic biological findings towards application. 98% of small-molecule and nearly 100% of large-molecule drugs are not able to cross the BBB (Pardridge, 2005). Furthermore, clinical studies reported by the largest companies involved in drug discovery and development indicate a continued low translation between first-in-man studies and approval of novel therapeutics, particularly in CNS-linked diseases, where the overall success rate is low, even though the clinical candidates have previously been tested in cellular *in vitro* or *in vivo* models (Dowden & Munro, 2019; Kola & Landis, 2004; Waring et al., 2015). This indicates the urgent need for alternative methods and implementation of appropriate testing strategies. One of the key determinants for the below-average success in targeting CNS disease is the non-standardized and often contradictory use of *in vitro* and *in vivo* test systems for characterizing the effects of potential therapeutic agents (small molecule compounds and biologicals, in particular antibodies). The reasons behind this phenomenon are complex and specific to the respective drug development program but include on- and off-target related toxicities, insufficient efficacy, inadequate validation of the disease-target linkage (poor target validation), and insufficient availability of the agent at the intended site of action in the brain. Accurate determination of efficacy of a test substance is a key readout parameter in early drug discovery projects. In addition, the establishment and validation of disease-relevant *in vitro* and *in vivo* models is essential to program success. If the intended compound cannot enter the brain, then efficacy cannot be achieved, regardless of the degree of target validation and failure is guaranteed. Therefore, data on the permeability of substances across the BBB are ultimately indispensable for estimating the availability and effectiveness of CNS drugs. However, some aspects have to be considered for a reliable prediction of the BBB permeability, e.g., species differences between humans and non-human primates/rodent models, as well as the substitution of brain capillary endothelial cells (BCECs) with peripheral tissue-specific endothelial cell (EC) sources. Cell sources with divergent functional characteristics, for example epithelial cell lines (e.g., Caco-2 or MDCK) or ECs of non-cerebral origin (e.g., HUVEC), provide another reason why these transport models are inadequate for the task at hand. Compounding this problem are the lack of regulatory guidelines to assist in the validation of new models for BBB permeation. Functionally relevant and validated human BBB models accurately predicting the transport of substances into the brain together with other methods such as serum binding, brain slice uptake, or brain homogenate binding assays will greatly reduce the requirement for *in vivo* testing of compound distribution and action in the brain and is highly relevant for all CNS-related toxicity, pharmacokinetic, pharmacodynamics and efficacy studies. A physiological and disease-relevant BBB model would act as a pre-screening platform to evaluate the capability of chemical or biological agents to penetrate or actively overcome the BBB. Only substances with the potential to cross the BBB would be taken into account to determine direct and indirect neurotoxic effects. Preclinical safety and efficacy studies for newly developed drug candidates could be a main field of application of human BBB models. Especially in the early phase of lead optimization, it is expected to induce an improved selection of the drug candidates but also an improved predictivity in later developmental stages. In 2012, the first BBB models derived from human-induced pluripotent stem cells (hiPSCs) were invented and are now reaching a level of validation that might make them suitable for utilization in preclinical drug development programs in the pharmaceutical industry. In addition, these models could provide insights into mechanisms of CNS diseases, which are often associated with general or specific pathophysiological alterations at the BBB.

In this review we discuss how hiPSC-derived BBB models compare to widely applied barrier test systems, such as Caco-2 and parallel artificial membrane permeability assay (PAMPA), as well as to BBB models from primary human or non-human BCECs and rodent *in vivo* models. In particular, we are focusing on the predictivity of the model types, the current technological status to generate hiPSC-derived BBB models and future trends in pharmaceutical development.

STRUCTURE AND FUNCTION OF THE BBB IN HEALTH AND DISEASE

The average adult human brain consists of ∼100 billion neurons and weighs around

1.5 kg with a volume of around 1.2 L. Although it constitutes only 2% of the total body weight, around 20% of the basal metabolic energy is consumed. Proper functioning of the vasculature supplying the brain with nutrients is required to maintain this high energy demand. The capillaries of the brain parenchyma have a length of around 600 km with a diameter of 7 μm (Keller, 2013; Wong et al., 2013). The circumference of these brain capillaries are lined with specialized ECs, representing the main component of the BBB and interacting with pericytes, astrocytes, neurons, microglial cells, and extracellular matrix components (ECM). The complex and dynamic association and communication of the elements led to the definition of the term neurovascular unit (NVU). The BCECs show specialized characteristics due to lack of fenestrations, higher mitochondria numbers, minimal pinocytotic activities, low rate of transcytosis, low expression of leucocyte adhesion molecules, higher pericyte coverage, special astrocyte end feet coverage, and the expression of dense tight junctions (TJs), as well as an increased expression of solute carriers and efflux transporters when compared to peripheral vasculature (Hawkins & Davis, 2005; Keller, 2013). This complex interaction of the cell types and functionalities make up the BBB. The main functions of the BBB can be divided into three subgroups, the physical-, metabolic-, and transport-barrier (Neuhaus & Noe, 2010). The BBB regulates and modulates biochemical and activated cellular traffic at the NVU thereby maintaining the homeostasis of the brain microenvironment (Abbott & Friedman, 2012).

Tight Junctions at the BBB

Entry of hydrophilic molecules and immune cells to the CNS is restricted by the presence of junctional protein complexes. These complexes are responsible for sealing the paracellular space and the degree of BBB tightness is determined via interaction of junctional proteins on neighboring BCECs. TJs limit the paracellular transport of substances to the brain and are also crucial for the polarization of the BCECs. The main transmembrane proteins involved in the formation of TJs are claudins, occludins, and junction adhesion molecules (JAMs). Adherence junctions (AJs) represent an additional type cellular junctions of BCECs and are a prerequisite for proper TJ formation. Especially claudin-5 is highly expressed in rodent BMECs. KO mice lacking claudin-5 are characterized by a size-selective leakage of the BBB. Occludin is highly enriched in CNS BCECs compared to other tissues. JAMs are known to regulate leucocyte extravasations, particularly JAM4 has been identified in the BBB of mice. The tight junction complexes are linked to the cytoskeleton via a series of adaptors and cytoplasmic accessory proteins such as zonula occludens (ZO)-1 and -2, cingulin, jacop, membrane-associated guanylate kinases, afadin, and 7H6 antigen. Additionally, the TJs interact with AJs. Main proteins of AJs are vascular endothelial cadherin (VE-cadh) and platelet EC adhesion molecule (PECAM-1), which are further linked to the cytoskeleton via catenins (Bauer, Krizbai, Bauer, & Traweger, 2014; Daneman & Prat, 2015; Liu, Wang, Zhang, Wei, & Li, 2012; Sweeney, Zhao, Montagne, Nelson, & Zlokovic, 2019; Weiss, Miller, Cazaubon, & Couraud, 2009). These TJs are responsible for barrier integrity which can for example be measured by the transendothelial electrical resistance (TEER). In frogs, average TEER values of around 1900 $\Omega*cm^2$ (Crone & Olesen, 1982) and in rats of around 1500 $\Omega*cm^2$ (Butt, Jones, & Abbott, 1990) were determined. Until now, no human *in vivo* reference TEER data is available.

Transporters at the BBB

The high energy demand of neurons is met by the selective entry of various monosaccharides, amino acids, and ions. Glucose is actively transported via glucose transporters-1 (GLUT-1) and GLUT-3. Small hydrophobic molecules, gases, and uncharged polar molecules can pass the membrane by simple diffusion. Short chain monocarboxylic acids, such as L-lactate, acetate, pyruvates, thyroid hormones, aromatic amino acids, and ketone bodies are transported via monocarboxylic acid transporters (MCTs) (Vijay & Morris, 2014). Proteins, such as insulin-like growth factors, vasopressin and transferrin, are transported into the brain via receptor-mediated transcytosis mechanisms (RMT). RMT mechanisms are specially investigated in the delivery of drugs to the brain, among the most importantly studied ones are the transferrin receptors (Tfr), low density lipoprotein receptors (LDLR) and insulin receptors (INSR) (Pulgar, 2018). Multispecific transporters of the ATP-binding cassette transporter families (ABCs) and solute carrier families (SLC) play crucial roles in regulating the entry of blood-delivered molecules, such as drugs into the brain. They recognize active endogenous compounds such as prostaglandins, leukotrienes,

and steroid hormones and prevent the brain from potential over-accumulation of these compounds. ABC transporters are responsible for the efflux of lipophilic and amphiphilic toxic compounds including several anti-inflammatory, anti-infectious, anti-depressant, and psychotropic agents (Strazielle & Ghersi-Egea, 2015). These transporters include P-glycoprotein (P-gp/ABCB1), breast cancer resistance protein (BCRP/ABCG2), and several multidrug-resistance associated proteins (MRPs/ABCCs). P-gp actively effluxes various xenobiotic compounds. P-gp, BCRP and MRPs show overlaps in substrate specificities thereby preventing the therapeutic entry of pharmaceuticals into the brain (Wevers & de Vries, 2016). BCRPs exclude therapeutics such as cytostatics and MRPs are known to efflux neutral organic drugs. Almost all transporters not belonging to the ABC transporter family belong to the SLC group, which consists of three main sub families. These carriers can be either unidirectional or bidirectional. The SLC22 subfamily includes charged organic transporters called organic anionic transporters (OATs) and organic cationic transporters (OCTs). The SLC21/SLCO forms a family of organic anion transport polypeptides (OATPs). SLCO members accept a broad range of substrates like environmental pollutants, nucleosidic antiviral drugs and non-steroidal anti-inflammatory agents. Members of the SLC15 family are required to transport endogenous di- and tri-peptides, peptidomimetic drugs (β-lactam) and antibiotics (Strazielle & Ghersi-Egea, 2015; Sweeney et al., 2019).

Disease- and Age-Associated Changes at the BBB

Altered BBB functions are shown in numerous brain disorders such as stroke, epilepsy, brain trauma, multiple sclerosis, Huntington´s disease, amyotrophic lateral sclerosis, schizophrenia (SCZ), Parkinson´s disease (PD), and Alzheimer´s disease (AD) (Greene et al., 2018; Neuwelt et al., 2011). Munji and co-workers recently analyzed BBB endothelial cells in mice suffering from neurological diseases including stroke, multiple sclerosis, traumatic brain injury, and seizures (Munji et al., 2019). BBB endothelial cells were analyzed using transcriptomics, and obtained gene expression profiles exhibited comparable gene expression changes in the context of different diseases. These data suggest that different diseases share common disease mechanisms affecting BBB functionality. One major result is that these changes induce loss of BBB characteristics and support a peripheral identity. Mice with seizures showed changes likely due to the increased metabolic activity of neurons highlighting that BCECs dynamically alter their properties in response to neural activity. Viruses such as poliovirus, adenovirus, Epstein-Barr virus, and West Nile virus can directly infect the BCECs while targeting JAMs or GLUTs. These infections can downregulate TJ proteins promoting chemokine production while host immune responses can attenuate BBB damage (Sweeney et al., 2019). In addition, the Zika virus infects and replicates in BCECs strain-independent, but changes in BBB permeability occurred strain-dependent (Leda et al., 2019). With the recent outbreak of the Coronavirus disease 2019 (COVID-19), researchers debate the need for neurological tissue models to understand the biology of SARS-CoV-2 infections. The brain has been reported to express angiotensin converting 2 enzyme (ACE-2) receptors, responsible for virus uptake, and brain autopsies of patients suffering from severe acute respiratory syndrome (SARS) presented with virus-infected brain tissue (Puelles et al., 2020). Furthermore, recent studies have proposed these manifestations in hospitalized patients affirming the neurotropic potential of the virus (Mao et al., 2020). Nevertheless, current data do not allow to conclude that SARS-CoV-2 can permeate across the BBB. It is known that the thickness of the basement membrane of the BBB increases during postnatal development and continually increases in aged animals. Other changes include gliofibrillar proliferations, loss of BCECs, decreased mitochondria/mitochondrial dysfunction, and region-specific alterations in cross-sections of capillary walls and lumens (Erdo, Denes, & de Lange, 2017; Goodall et al., 2018). Notably, interspecies differences in these phenotypes exist: Recent studies in mice revealed ultrastructural changes of the BBB of mice with increasing age, showing an enhanced thickness and lipid accumulations compared to the human BBB (Ceafalan et al., 2019). Some studies using advanced magnetic resonance imaging were able to quantify regional BBB permeability in living human brains, showing an age-dependent BBB breakdown in the hippocampus (Montagne et al., 2015). In this article we will focus on disease-associated alterations at the BBB in AD. AD is the most common cause of dementia with a

steep increase of 5.8 million Americans diagnosed with AD with around 122,019 deaths in 2018 compared to previous years ("2020 Alzheimer's disease facts and figures," 2020). AD is characterized by impaired cognitive functions. The two main pathologies in AD affected brains are the formation of amyloid β (Aβ) plaques and tau tangles. A "two hit hypothesis" was suggested to explain the etiology of AD: Initially, damage occurs at the blood vessels resulting in BBB dysfunctions which in turn leads to Aβ accumulation in the brain and neurodegeneration. Studies from AD *post mortem* brains applying immunohistochemistry and immunoblotting show reduced TJ protein expression and capillary leakages of blood derived proteins, in particular albumins, immunoglobulins, thrombins, iron-containing proteins, and fibrinogens in brain areas with increased plaque depositions. Imaging studies show leaky BBBs in AD patients suggesting this phenotype to be an early biomarker for AD (Neuwelt et al., 2011; Sweeney, Sagare, & Zlokovic, 2018). In addition to non-specific leakages, nonfunctional BBB transport can drive AD pathology. Molecular changes such as low levels of GLUT-1 expression in BCECs were shown to result in diminished glucose transport (Erickson & Banks, 2019). The efflux transporters P-gp and the receptor LRP-1 are major regulators of Aβ CNS levels. Patients with AD show decreased protein levels of LRP-1 and P-gp. This led to the hypothesis that inefficient efflux at the BBB can lead to progression of AD via reduced clearance of Aβ from brain parenchyma. LRP-1 surface receptors on BCECs are responsible for Aβ clearance. They were shown to be diminished in AD brain microvessels leading to reduced clearance of Aβ promoting intracerebral accumulations. AD mouse models with an EC-specific knockout of LRP-1 show increased levels of soluble brain Aβ and severe learning and memory deficits while P-gp deficiency decreases Aβ clearance rates (Banks, 2016; Desai, Monahan, Carvey, & Hendey, 2007). In addition, BBB breakdown also leads to the increased uptake of inflammatory mediators like cytokines, chemokines, peripheral leukocytes, and CD4+T cells into the brain parenchyma thereby accelerating disease progression. Red blood cell extravasation, as well as infiltration by peripheral macrophages and neutrophils reported in AD *post mortem* studies further suggest innate immune system activation in the brain contributing to the pathophysiological changes (Engelhardt, Vajkoczy, & Weller, 2017; Nishihara et al., 2020; Sweeney et al., 2019). ABCA7, which shares a significant sequence homology to ABCA1, mainly promotes cholesterol and phospholipid transport across membranes, but involvement in phagocytic activity and Aβ clearance pathway was also shown in mouse models (Jehle et al., 2006; Kaminski et al., 2000; Kim et al., 2013; Sakae et al., 2016). ABCA7 levels were found to be increased in AD brains due to a compensatory regulation as suggested by the authors (Karch et al., 2012; Vasquez, Fardo, & Estus, 2013). Further, ABCA7 was identified by GWAS as a susceptibility locus contributing to the late-onset form of AD and loss-of-function variants of ABCA7 were reported for AD patients (Almeida, dos Santos, Trancozo, & de Paula, 2018; Hollingworth et al., 2011; Lambert et al., 2013; Steinberg et al., 2015; Vasquez et al., 2013). Recently, it was shown that an ABCA7 polymorphism (rs3764650) within apolipoprotein E carriers of a homozygous risk haplotype (APOE-ε4) results in memory impairment and functional network connectivity in AD patients (Chang et al., 2019). Many mechanisms underlying the onset, progression, and severity of CNS diseases are not completely understood, in particular with regard to BBB functionality (Profaci, Munji, Pulido, & Daneman, 2020). Furthermore, it is not yet understood if the disease-specific BBB degradation is cause or effect of neurological diseases.

APPLICATION OF BBB MODELS IN PRECLINICAL DRUG DISCOVERY

Value of BBB Models for Drug Discovery and Development

The increasing age of the world's population is significantly associated with a growing number of CNS-related diseases, including AD, PD, brain cancer, or stroke, resulting in an urgent need to develop cost-effective packages of medical and social care. A major problem in the CNS drug discovery process is the BBB penetration of effective therapeutic agents in sufficient amounts (Ghose, Herbertz, Hudkins, Dorsey, & Mallamo, 2012). This is one of the reasons for low success rates in CNS drug discovery, resulting in halted drug development programs in pharmaceutical companies for these indications. The increasing number of patients, however, is reflecting the dramatic need of effective drugs: Only for dementia it is predicted that the number of patients in the United Nations population will double every

20 years from 36 million people in 2010 to 115 million in 2050 (Prince et al., 2013). In order to increase the market release of CNS drugs, two major issues need to be overcome in preclinical development: First, the understanding of physicochemical and structural drug characteristics, which correlate with good penetrability needs to be improved; second, better translational models have to be provided to evaluate BBB penetration capability and efficacy of CNS therapeutics. BBB *in vitro* models are useful tools to study BBB permeability and to predict pharmacological availability of drugs, such as small molecules, antibodies, proteins, and peptides. The pharmaceutical industry seeks for standardized and predictive human BBB *in vitro* models in order to differentiate between CNS active and non-active compounds early on in the drug development process.

Currently Applied BBB Models in Early Stage Drug Discovery

Due to the high demand for test systems in basic and preclinical research of drug development and transport studies, a range of different BBB models have been implemented. Besides the *in silico*, acellular *in vitro* and *in vivo* models, numerous cell-based BBB models have been developed. However, standardized models based on immortalized human cell lines show only moderate TJ expression and possess low barrier integrity, which is detected through TEER values below 150 Ω*cm^2 (Deli, Ábrahám, Kataoka, & Niwa, 2005). In comparison, the TEER values in animal experiments reached average values of more than 1500 Ω*cm^2 at the BBB (Butt et al., 1990; Crone & Olesen, 1982). These values are valuable benchmarks from *in vivo* experiments for the *in vitro* model validation, but it should be highlighted that also significantly lower and higher values have been measured in these reports. The availability of human primary BBB cells and healthy human brain tissue is very limited and ethically troublesome. Isolated primary cells change their characteristics rapidly during *in vitro* cultivation. Furthermore, the limited possibility to subculture or cryo-preserve primary cells make them unsuitable for a standardized industrial application. Most widely used primary BBB models in the pharmaceutical industry are based on bovine and porcine brain microvascular endothelial cells (BMECs), but the isolation and cultivation processes are very labor intensive and lead to variable results (Reichel, 2006). In this regard, it should be mentioned that the isolation of pure BCECs is quite difficult. Most procedures yield BMECs and not BCECs, since many protocols end up with a mixture of BCECs with other cells from the microvasculature such as brain arteriole or venule endothelial cells. Furthermore, due to the increasing knowledge of BBB-specific species differences, reproducible human *in vitro* models become more and more important. Most prominent differences are known in the expression of the transporters P-gp *versus* MDR as well as claudin subtypes in rodents and humans (Aday, Cecchelli, Hallier-Vanuxeem, Dehouck, & Ferreira, 2016). By aid of quantitative targeted absolute proteomics (QTAP) of human brain transporters and receptors, it was shown that P-gp expression in humans is 2.33-fold less compared to mdr1a expression in mice. More than 2-fold differences in protein expression were also detected for multidrug resistance-associated protein 4 (ABCC4/MRP4), monocarboxylate transporter 1 (MCT1/SLC16A1), l-type amino acid transporter (LAT1/SLC7A5), and organic anion transporter 3 (OAT3/SLC22A8) in comparative studies of human and rat BBBs. The amounts of ABCG2/BCRP, SLC2A1/GLUT-1, and INSR were similar in both species. These data together with others highlighted that the expression of ABCG2/BCRP was higher than the one of ABCB1/P-gp in humans (Shawahna et al., 2011). Moreover, the specific transporter MRP could not be detected in humans but is reported to be expressed in the rodent BBB. In addition, the expression level of LAT1/SLC7A5 was decreased 5-fold compared to mice (Hoshi et al., 2013; Uchida et al., 2011). Thus, intraspecies differences have an important impact on reproducibility and translation of drug transport efficacy studies (Aday et al., 2016; Bhalerao et al., 2020). Beside the expression of transporters, differences in the presence and quantity of tight junction proteins as well as receptors are reported between humans and rodents. The expression of claudin-3, -5, and -12 was reported to be most relevant in mouse models (Krause et al., 2008; Pfeiffer et al., 2011) until recently the role of claudin-3 and -12 for the barrier function of the BBB in mice was questioned (Castro Dias, Coisne, Baden et al., 2019; Castro Dias, Coisne, Lazarevic et al., 2019). On the contrary, the expression of claudin-1 was only evident at the human BBB (Berndt et al., 2019; Liebner et al., 2000). Claudin-5 expression dominates the BBB *in vitro*, but this

does not reflect the *in vivo* situation. The expression profile of mouse and human TJ proteins *in vivo* is much more complex, but their complexity is largely lost under *in vitro* conditions (Berndt et al., 2019). In general, the properties of BCECs *in vivo* are better comparable to primary human BBB models than to immortalized cell lines (Bagchi et al., 2019). Due to the simple and cost-efficient usage, cell-free PAMPA assays and non-cerebral cell lines, such as Caco-2 or kidney epithelial cells (MDCK), are widely utilized in BBB assays. The MDCK cell line for example is characterized by a tighter barrier and has therefore been used to rank order passive permeation characteristics. Despite its routine use, permeability data obtained by the intestinal cell line Caco-2 cannot be used to evaluate CNS penetration, due to lacking tissue-specificity and, therefore, significance (Reichel, 2006). PAMPA assays are based on transwell systems containing a lipid artificial membrane in order to predict BBB permeation. PAMPA represents a predictive surrogate test for transcellular passive diffusion. Using this assays, compounds can be classified into high, low or uncertain BBB permeation categories. In general, PAMPA assays are considered a high throughput, low cost, and reproducible method. Nevertheless, active transport processes cannot be examined using PAMPA assays, resulting in an overestimation of *in vivo* penetration of tested substances (Bicker, Alves, Fortuna, & Falcao, 2014). In summary, no currently applied *in vitro* test system is able to mimic the *in vivo* complexity of the BBB and its properties (Bicker et al., 2014). As long as the ideal model, representing all critical aspects of BBB penetration characteristics at the same time, is not available, industry-driven drug development programs rely on the combination of several models to separately investigate the passive permeability and specific transport processes of compounds (Reichel, 2006). An alternative to avoid the aforementioned problems and to provide standardized human BBB models by the use of reproducible conditions, could be the application of hiPSC-derived test systems.

CURRENT TECHNOLOGICAL STATUS IN HIPSC-DERIVED BBB MODELING

Comparison of Differentiation Methods and Cellular Characterization

Within the last eight years a variety of different hiPSC-derived BBB models have been established, following alternative differentiation strategies and test system complexities. As summarized in Table 1, numerous methods of successful *in vitro* hiPSC differentiation, under reproducible conditions, were already developed. The BCECs were examined for the presence and the functionality of endothelial-specific markers, as well as specific transporters using transcriptional and proteomic methods. The most extensively used protocol for the derivation of BCECs is the co-differentiation protocol developed by Lippmann and colleagues (Lippmann et al., 2012). The protocol comprises of the following main steps: Firstly, the hiPSCs are differentiated to neural cells and ECs, followed by selective maturation of ECs and finally the elimination of neural subtypes via sub-culture onto collagen IV and fibronectin ECM. Furthermore, retinoic acid (RA) was identified as a suitable component in the enhancement of BBB phenotypes (Lippmann, Al-Ahmad, Azarin, Palecek, & Shusta, 2014; Stebbins et al., 2016; Wilson, Canfield, Hjortness, Palecek, & Shusta, 2015). The protocol has been reproduced by us and various other groups (Appelt-Menzel et al., 2017; Appelt-Menzel, Cubukova, & Metzger, 2018; Katt, Xu, Gerecht, & Searson, 2016; Yamashita, Aoki, Hashita, Iwao, & Matsunaga, 2020). Updates have been made to the original method in accelerating the differentiation process, use of serum-free media or efforts to eliminate additional purification steps (Hollmann et al., 2017; Neal et al., 2019; Ribecco-Lutkiewicz et al., 2018). Inhibition of TGFβ pathway induced the expression of cellular markers and cell-specific characteristics, as well as reduced damages induced by freezing/thawing processes (Yamashita et al., 2020). The use of embryoid bodies (EBs) and co-culture with rat glioma cells or conditioned medium has been explored (Minami et al., 2015). A shift from the co-differentiation protocol to direct BCECs generation was achieved by using chemically defined factors (Grifno et al., 2019; Praca et al., 2019; Qian et al., 2017). Recent advancements have been focused on the overexpression of transcription factors in upregulating BBB phenotypes (Roudnicky et al., 2020). Here, Roudnicky et al. identified 17 transcription factors, including among others TAL1, SOX7, SOX18, ETS1, and LEF1, to improve the direct differentiation of vascular endothelial cells and analyzed them using gain-of-function assays (Patsch et al., 2015; Roudnicky et al., 2020).

Table 1 Overview of the Differentiation Methods to Generate hiPSC-derived BCECs and Comparison of the Cellular Characteristics

Strategy	Co-differentiation						Directed differentiation		Transcription factor overexpression	EBs + Co-culture
Original reference	Lippmann et al. (2012)	Lippmann et al.,(2014)	Hollmann et al.,(2017)	Ribecco-Lutkiewicz et al. (2018)	Neal et al. (2019)	Yamashita et al. (2020)	Qian et al. (2017)	Praca et al. (2019)	Roudnicky et al. (2020)	Minami et al. (2015)
Days required	~13	~13	~8	~21	~8	~13	~10	~15-20	~6	~14
Purification step	+	+	+	-	+	+	-	+	-	+
TEER (Ω*cm^2) monoculture	~200	~3000	~1000-4500	~300-800	~2000-8000	~1500-4000	~3000	~50-60	~20-25	~22-28
TEER (Ω*cm^2) co-culture (cells/ conditioned medium)	~1500	~5000	~6500	~1000-1500	~9000-10,500	N/A	~4000	~50-60	N/A	~55
Tube formation *in vitro*	+	N/A	N/A	N/A	N/A	+	+	N/A	N/A	+
Marker expression (immuno-fluorescence)	Claudin−5, GLUT−1, occludin, PECAM−1, P−gp, VE−cadh, von Willebrand Factor (vWF), ZO−1	BCRP, claudin−5, GLUT−1, MRP−1, occludin, PECAM−1, P−gp, VE−cadh	Claudin−5, GLUT−1, occludin, PECAM−1, VE−cadh	Claudin-5, GLUT−1, occludin, P−gp, PECAM−1,vWF, ZO−1	Claudin−5, GLUT−1, occludin, PECAM−1, VE−cadh	BCRP, claudin−5, GLUT−1, occludin, P−gp, VE−cadh, ZO−1	BCRP, claudin−5, GLUT−1, occludin, MRP−1, PECAM−1, P-gp, VE−cadh, vWF, ZO−1	Claudin−5, GLUT−1, occludin, PECAM−1, P−gp, Tie−2, VE−cadh, vWF, ZO−1	Claudin−5, occludin, VE−cadh, ZO−1	PECAM−1, vWF

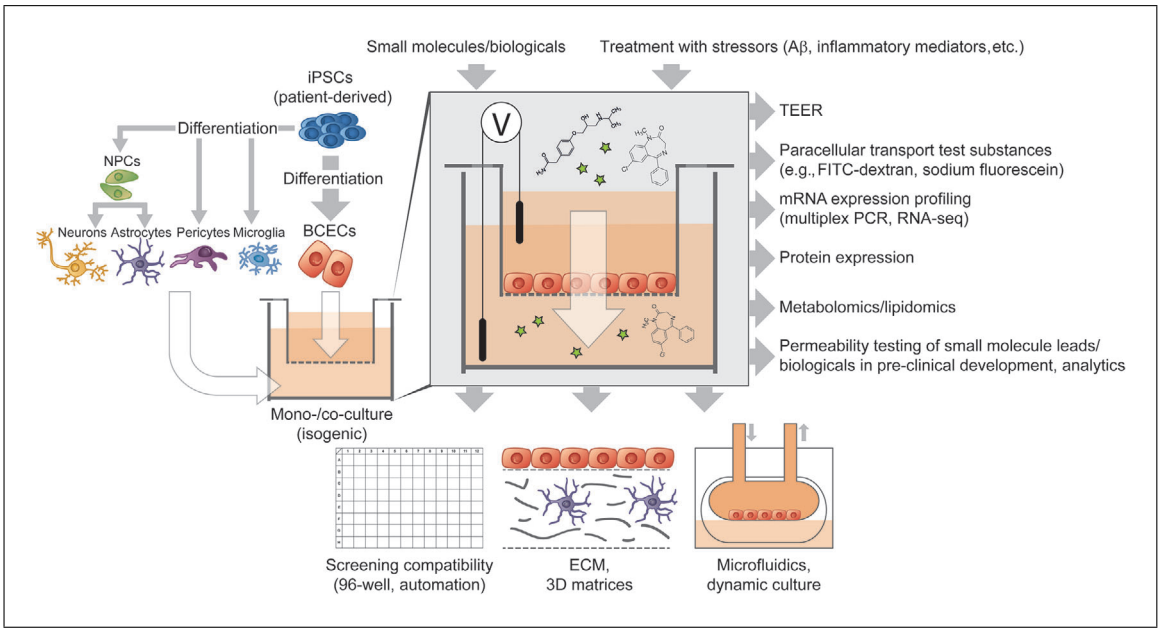

Figure 1 Features of hiPSC-derived BBB models. BCECs are differentiated from healthy or diseased hiPSCs and seeded in the apical compartment of a transwell plate. Patient-specific hiPSC-derived neurons, astrocytes, pericytes, and/or microglia in the basolateral compartment can be used to recapitulate the cellular composition of the NVU and to establish isogenic models. In such a setup, various read-outs are possible either interrogating the BCEC barrier properties, such as TEER measurements or analysis of paracellular transport of test substances is possible. Furthermore, the permeability of small molecule leads and biologicals in preclinical development can be tested. Moreover, additional parameters such as RNA or protein expression levels, as well as metabolomics and lipidomics profiles can be studied. In addition, both compartments can be used to treat or challenge the barrier with soluble factors and to imitate disease-specific environmental stressors (e.g., Aβ or inflammatory mediators). Of note, this setup also allows for the application of HTS formats (up to 96-well transwell plates) and at least partially automatization, dynamic/microfluidic culture resulting in BCEC stimulation by shear stress or the usage of advanced extracellular matrix constituents and three-dimensional structures.

Types of hiPSC-Derived *In Vitro* BBB Models

Static transwell models in mono- and co-culture

Transwell-based models mainly consist of BCECs cultured on a semipermeable cell culture membrane, providing a separated apical and basolateral culture compartment. These models are easy to use; the main advantage is the suitability for unsophisticated co-culture, TEER measurement, and permeability studies. This model is static and the plastic insert membrane acts as an extrinsic barrier, which can be considered as a drawback (Gastfriend, Palecek, & Shusta, 2018). Depending on the protocol used for differentiation, BBB models using these mono-cultures led to varying TEER values (brief summary provided in Table 1) (Delsing et al., 2018; Hollmann et al., 2017; Katt, Linville, Mayo, Xu, & Searson, 2018; Lippmann et al., 2014; Lippmann et al., 2012; Lippmann, Al-Ahmad, Palecek, & Shusta, 2013; Neal et al., 2019; Qian et al., 2017; Ribecco-Lutkiewicz et al., 2018; Stebbins et al., 2016). Recent comparisons of these models have been made to porcine-based systems, resulting in similar drug permeability data for a set of 23 CNS targeting compounds with a correlation coefficient of $R^2 = 0.8$. In addition, activity differences of transporters were highlighted for efflux transporters, as well as GLUT-1 and LAT-1 (Di Marco et al., 2020). Co-culture with pericytes or hiPSC-derived ACs and neurons increased TEER values in transwell models by 30%, while monocultures had values around 3000 Ω*cm^2 (Qian et al., 2017). HiPSC-derived BCECs and PCs co-cultures enhanced BBB phenotypes with reduced levels of transcytosis (Stebbins et al., 2019). Previously, we have analyzed a set of ten different BBB culture models using hiPSC-derived BCECs, multipotent (fetal) neural stem cells, ACs, and PCs. The most complex culture models were able to investigate the distinct upregulation of typical BBB genes, as well as significant increase of TEER compared to the mono-cultures (Appelt-Menzel et al., 2017; Appelt-Menzel et al., 2018). Studies like ours confirmed that the combination of cell types of the NVU enhances barrier properties

compared to mono-cultures of hiPSC-derived BCECs. Ribecco and colleagues developed the first syngeneic stem cell model to study receptor-mediated transcytosis and its application in evaluation of antibody-based BBB carriers. The mono-cultures yielded TEER between 300 and 800 $\Omega*cm^2$, while with AC conditioned medium the TEER is elevated to $\sim$1000 $\Omega*cm^2$ (Ribecco-Lutkiewicz et al., 2018). Transwell models based on hiPSC-derived BCECs can generally also be used to mimic aspects of neurological diseases, as already shown for example for cerebral ischemia (Kokubu, Yamaguchi, & Kawabata, 2017; Page, Raut, & Al-Ahmad, 2019), Huntington's disease (Lim et al., 2017), MCT8 deficiency (Vatine et al., 2017) or cerebral amyloid angiopathy (CAA) and AD (Blanchard et al., 2020). An overview of hiPSC-derived transwell models is provided in Figure 1.

Planning and performing co-culture experiments face challenges like choosing the ideal media composition for every cell type of the NVU to ensure e.g., viability of co-cultured cells and the maintenance of cell type-specific markers. Considering the effect of cell maturity to signaling pathways, another aspect is finding the optimal timing of bringing together several cell types or various differentiation protocols to one timepoint.

Microfluidic models

Compared to transwell models the microfluidic test systems offer benefits like imitation of fluid flow and shear stress conditions found in physiological situation; however, the scalability and requirement of special expertise is a drawback when using these models for drug discovery applications (Gastfriend et al., 2018). Perfused hydrogels show that hiPSC-derived BCECs form confluent 3D monolayers with barrier integrity up to 3 weeks in culture. Perfusion of the cell-lined channels in low stress conditions stabilizes the barrier integrity over non-perfused controls, hence, indicating that perfusion with shear stress enables long-term barrier functions due to mechanical cues and continuous medium circulations. As a downside, angiogenesis was reported in the perfused channels, presumably due to lack of other NVU cell types and their secreted factor or cell-cell contacts (Faley et al., 2019). DeStefano and colleagues have shown that shear stress does not significantly affect the proliferation, rate of apoptosis, elongation, alignment, and gene expressions in hiPSC-derived BCECs (DeStefano, Jamieson, Linville, & Searson, 2018). Recently, hiPSC-derived BCECs co-cultured with ACs in perfused microfluidic channels were reported to maintain *in vivo*-like TEER values for 12 days, whereby the authors concluded that shear forces were not essential for the establishment of strong intercellular junctions but required to stabilize barrier integrity over time (Wang, Abaci, & Shuler, 2017). HiSC-derived BCECs co-cultured with rat primary ACs in a pumpless microfluidic platform for 10 days showed maintained TEER of $\sim$2000 $\Omega*cm^2$. This system was used as a drug candidate screening platform (Wang et al., 2017). A first-of-a-kind human hypoxic BBB chip model permitting analysis of BBB penetrating peptides and TfRc shuttling mechanisms has been described by Park and colleagues (Park et al., 2019). Channels with cylindrical geometry mimicking the three-dimensional architecture of the BBB have been introduced in proof-of-concept studies (Grifno et al., 2019; Katt et al., 2018; Linville et al., 2019).

Isogenic models

Canfield and colleagues reported for the first time that an NVU transwell model with hiPS-derived neurons, ACs, and BCECs in physiological cell ratios facilitated the investigation of the BBB. The endothelial stimulation by neurons, as well as ACs to the co-culture models induced barrier tightening, tight junction continuity and elevated TEER values, while P-gp efflux transport activity remained unchanged. The main limitation in this study was the absence of isogenic PCs (Canfield et al., 2016; Xiang, Andjelkovic, Wang, & Keep, 2017). In order to overcome the limitation of the previous study, the authors further included the effects of isogenic PCs in a new study, which led to the reduction of the rate of non-specific transcytosis. In this model, the authors noticed no differences in efflux activity compared to mono-cultures, although the co-cultures demonstrated barrier tightening and significant increases in expression of junctional proteins, especially occludin (Canfield et al., 2019). Patel and colleagues investigated the variability on phenotype and cell yield after differentiation in isogenic BBB models using asymptomatic patient samples. Differences in differentiation efficiencies of hiPSC-derived BCECs were noticed for PECAM-1, GLUT-1, and P-gp expression and drug efflux pump activities. The observation of such differences suggest interindividual polymorphisms or sexual dimorphisms; therefore, these observations must be taken into consideration for isogenic disease model

development (Patel, Page, & Al-Ahmad, 2017). Recently, novel isogenic NVU chip models are developed supporting co-culture of hiPSC-derived BCECs and ACs. These chip models support media flow, which can be beneficial in recapitulating physiological conditions (Motallebnejad, Thomas, Swisher, & Azarin, 2019).

Preclinical Permeability Prediction and Potential Application of hiPSC-derived BBB Models

Robust and standardized hiPSC-derived BBB *in vitro* models could be valuable tools for preclinical drug-discovery because they fulfill two fundamental important criteria: They demonstrate physiological BBB relevant *in vivo*-like characteristics and simultaneously are compatible with the high-throughput demands of the pharmaceutical industry. Upscaling technologies using stirred tank bioreactors, spinner or suspension flasks are already routinely used for standardized hiPSC maintenance, as well as differentiation (Ackermann et al., 2018; Halloin et al., 2019; Kropp et al., 2016; Schwedhelm et al., 2019; Silva et al., 2015). On this basis, a high-throughput drug-compatible BBB assay should be feasible. For every developed CNS targeting compound, but also for substances acting in the periphery in order to exclude CNS-mediated side effects, it is mandatory to determine the brain targeting efficacy, as well as the toxicity. BBB *in vitro* models can be applied to perform transport studies, visualize transport routes, analyze drug transporter functionalities, perform drug interaction, or targeting studies, ensure pharmacological safety, examine disease-relevant BBB functions and conduct drug discovery studies (Prieto et al., 2004). The process of developing novel therapeutic agents follows a strategy that is based on the same principle across the entire pharmaceutical industry. Usually, the process of drug discovery is divided into different phases sequentially including target identification, hit identification, lead identification, and lead optimization. There are, however, differences between companies, in particular concerning the detailed organization of the various phases of the drug discovery and development processes. For compounds destined to act within the CNS, the penetrability of the BBB is an important additional property, which is typically investigated during lead generation (Reichel, 2006). A typical high-throughput screen (HTS) results in up to 1% hits of the initial screening library, which after hit validation (comprising of confirmation of single dose hits from the primary screen in replicates, assessment of compound identity and purity) and hit qualification (comprising of *in vitro* potency and selectivity towards related targets, early structure-activity relationships, physiochemistry, pharmacokinetics, and toxicology) narrow down to a handful of lead series (ideally providing a chemically diverse lead-like compound series with sufficient potential for chemical optimization). The characterization of lead compounds and lead series involves *in silico* tools, *in vitro* models and *in vivo* studies to examine pharmacological, pharmacokinetic and early toxicological properties. In programs aiming for the development of CNS therapeutics, the pharmacokinetic studies involve tests for CNS penetration, ranging from *in silico* classification systems, *in vivo* permeability assays, and for selected compounds the determination of brain-to-plasma or CSF-to-plasma ratios *in vivo*. Leads will serve as starting points for iterative steps of chemical optimization with the goal to increase *in vitro* potency and selectivity and to improve *in vivo* pharmacokinetic and pharmacodynamics properties by rational medicinal chemistry efforts. Ultimately, compounds need to be efficacious in relevant animal disease models in order to be progressed. The most promising compounds of these lead series which show *in vivo* efficacy are thoroughly characterized in order to identify the most critical properties and hence, the determining characteristics for the direction of chemical optimization in the subsequent drug development phase. The transition from discovery to development represents a major decision point, as all further activities on the candidate drug will demand a substantial commitment in terms of manpower, resources and budget. Drug development begins with a preclinical phase, which investigates in great detail the metabolism and pharmacokinetics, as well as the toxicology and safety profile of the compound in several (rodent and non-rodent) animal species in order to prepare the testing of the compound into human studies. Current strategies aim at combining elements of drug discovery and development phases earlier in the process to optimize safety and efficacy parameters. Clinical studies establish the safety and pharmacokinetics of the compound in humans (phase I study), from which the dosing scheme is derived for the subsequent phase II and phase III studies to demonstrate clinical efficacy in multi-center trials. To prevent

misinterpretations and incorrect predictions resulting from species differences with regard to the expression of transporters, receptors and TJ proteins which influence BBB permeability, predictive human *in vitro* BBB models should also to be taken into account in drug discovery studies to complement the results (Aday et al., 2016). Obviously, the importance of the BBB and their integrity should be considered to estimate the penetration capacity of newly developed drugs and their metabolites, but on the other hand should also not be ignored in neurotoxicity screenings. The combination of predictive and robust BBB models with downstream located neurological systems can be a future testing strategy of choice. To increase standardization and simplicity, as well as to reduce workload and cell culture costs, cryopreserved hiPSC-derived BCECs can potentially be utilized for customer-specific applications (Wilson, Faubion, Hjortness, Palecek, & Shusta, 2016). The cryopreservation and banking of hiPSC-derived BCECs or respective progenitors will be a future trend for a standardized application of hiPSC-derived BBB models in drug discovery. Several pan-European, US, and Japanese strategies led to the establishment of sophisticated cell banks to address this need over the last decade and could serve as a source for high-quality reference lines used for hiPSC-derived BCEC progenitor generation (De Sousa et al., 2017; McKernan & Watt, 2013; O'Shea, Steeg, Chapman, Mackintosh, & Stacey, 2020).

In Vitro-In Vivo Correlation of Permeability Data Obtained from hiPSC-derived BBB Models

Especially *in vitro* models derived from human cell lines and stem cells have been demonstrated as useful tools for preclinical drug discovery (Aday et al., 2016). During the last years, different hiPSC-derived BBB *in vitro* models were evaluated for their applicability to predict drug permeability of novel treatment modalities, providing promising future concepts for CNS drug development. After differentiation of hiPSCs into BCECs and model characterization, the group of Lippmann et al. correlated in 2012 for the first time permeability values with *in vivo* rodent brain uptake measured by *in situ* brain perfusion, confirming high correlative values for the transport prediction of small molecules with a correlation coefficient of $R^2 = 0.98$ (Lippmann et al., 2012). Le Roux and colleagues differentiated hiPSCs in accordance to protocols described by Lippmann et al. and included an additional puromycin-based selection process for EC purification. The morphology of the differentiated cells was comparable to primary human BMECs and expression of ZO-1, as well as claudin-5 was confirmed on protein level. Except for PECAM-1 and ABCB1, the mRNA expression level of transporters and receptors including ABCC1, ABCG2, TfRc, and INSR was similar to primary cell cultures. Furthermore, they compared BBB permeability of eight clinical positron emission tomography (PET) radioligands in comparison to the human BBB *in vivo*, showing a highly significant correlation ($R^2 = 0.83$, $P = 0.008$) (Roux et al., 2019). With another panel of 18 compounds, the group aimed to further evaluate the ability of the hiPSC-derived BBB *in vitro* test systems to distinguish between CNS and non-CNS drugs. Indeed, the permeability values between both groups significantly differed, showing high values of CNS active drugs (mean Papp $= 30.1 \pm 4 \times 10^{-6}$ cm/s) and only low transport rates of non-CNS drugs (mean Papp $= 2.1 \pm 0.6 \times 10^{-6}$ cm/s), but a relationship between permeability and physicochemical properties of the compounds could not be established. In this context, Papp describes the apparent permeability coefficient and is often used to describe transport velocities of drugs. In intestinal models this value correlates with the absorption. Moreover, species-related transport differences could be confirmed comparing the transport data to an *in vitro* BBB model based on primary rat cells. Prediction of *in vivo* human BBB permeability was also investigated by Ohshima and colleagues using hiPSC-derived mono- and co-culture models (Ohshima et al., 2019). Transporter expression profiles of the hiPSC-derived BCECs was similar to that of human primary BMECs, barrier integrity with TEER values $>1000\ \Omega*cm^2$ and expression of relevant TJ markers, such as claudin-5, ZO-1, and occludin was confirmed. In detail, the expression of transporters and other tissue relevant markers was not affected by co-cultures with pericytes, astrocytes, or neurons. Especially the expression level of P-gp/ABCB1 was similar between the different BBB set-ups, but significantly lower than in the analyzed Caco-2 models. Drug permeability assays yielded a better correlation between hiPSC-derived BBB models and data obtained from *in vivo* assays than rat BBB models or Caco-2 assays. Antibody-triggered

RMT was studied by Ribecco-Lutkiewicz and colleagues (Ribecco-Lutkiewicz et al., 2018). The applied hiPSC-derived BBB model expressed RMT-associated receptors, as LDLR, TfR, INSR, or TMEM30A (receptor for BBB-crossing antibody FC5) and allowed the discrimination of species-selective antibody-mediated transcytosis mechanisms. Correlation of Papp values derived from specific antibody transport and the apparent CNS exposure in rat [simultaneous pharmacokinetic measurements of antibodies in cerebrospinal fluid (CSF) and in the serum] was significantly high with $R^2 = 0.96$.

REGULATORY REQUIREMENTS FOR PRECLINICAL DRUG TESTING AT THE BBB

Regulatory Status Quo

A related key problem within the domain of drug permeability testing at the BBB is that until now no regulatory guidelines for the validation of *in vitro* models are available. An exception are Caco-2 models mimicking the gut epithelium (Volpe, 2011; Volpe et al., 2007). Already in 1993, the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM) defined the prevalidation and validation of non-animal methods for predicting the penetration of chemicals through the BBB as a current priority (*https://cordis.europa.eu/article/id/10815-alternative-methods-in-biosciences*, accessed on 21.04.2020). Furthermore, within the report of the ECVAM Workshop 49, the need for *in vitro* models to determine BBB transport was again underlined. As there are large quantitative and qualitative differences in BBB systems, EURL ECVAM defined the presence of a restrictive paracellular permeability, the presence of a physiologically plausible cell architecture/morphology, the expression of *in vivo* relevant transporter mechanisms, as well as the simplicity of cell culture as minimal requirements for useful BBB models (Prieto et al., 2004). To sum up the recommendations for the evaluation of the performance of promising *in vitro* models, it was suggested to use an appropriate and defined set of selected compounds to better characterize and standardize the models. To define acceptance criteria of *in vitro* models, the expression of BBB relevant (active) transporters has to be compared to the *in vivo* situation. To ensure the comparability and model standardization, all applied methods have to be defined in standard operation procedures. Moreover, a more precise definition of the abovementioned minimal requirements for model characterization is required, stressing in particular the importance on studying cellular polarity and the restriction of paracellular transport. Model performance has been evaluated by analyzing influx and efflux rates, as well as barrier integrity. All obtained permeability and CNS toxicity data should be provided for setting up an EURL ECVAM database. Endpoints for model validation prior to substance testing have to be defined for valid BBB *in vitro* models. This includes for example the specification of permeability values describing the paracellular tightness of the barrier as well as the transport velocities of BBB relevant reference drugs. Furthermore, the definition of relevant substances, as well as optimal testing concentrations for the (pre-)validation of BBB models are missing, thereby providing an adequate model quality control. Moreover, this topic remains challenging due to the insufficient availability of human *in vivo* data. Nevertheless a wide acceptance of a validated human BBB *in vitro* model is prognosed, urging the supply of a guidance provided by regulatory authorities.

Requirements in Preclinical Drug Discovery

To develop more effective neuropharmacological drugs with the capability to cross the BBB, standardized, robust and highly predictive humanized BBB models should be applied to determine the penetration capacity, as well as to understand the related expression and functionality of transporters at the BBB. The results obtained by use of adequate human BBB test systems should valorize the toxicological results generated in animals, in order to improve the outputs of toxicity tests and to enhance the evaluation of risk and safety in comparison with clinical data in humans (Cecchelli et al., 2007). A challenge to be fulfilled for permeability screenings is the application of *in vitro* models that recapitulate essential aspects of the *in vivo* physiology of the BBB and are compatible with the high-throughput requirements of the pharmaceutical industry (Bicker et al., 2014). In order to evaluate efficiently the large numbers of compounds generated by the pharmaceutical and chemical industry, assays have to be compatible to 96- or 384-well formats, allowing at least a partial automatization of the experimental workflow and resulting data analysis. Furthermore, a simplified handling of the test systems should be feasible, including an easy

assay procedure with well-defined ready-to-use components/reagents ("mix-and-ready") and robust readout parameters using state-of-the-art detection technologies. An appropriate turnaround time ensures the milestone-based compound progression. Beside time efficiency, the assay costs have to be justifiable. A successful usage of hiPSC-derived BBB models in preclinical testing requires the availability of robust *in vitro* test systems, characterized by the presence of a physiologically relevant morphology and cellular polarity, a reproducible permeability of reference compounds, an adequate screening capacity, and the expression of complex tight junction proteins and transporters, as well as their functionality (Cecchelli et al., 2007). To avoid misinterpretations of preclinical toxicity and bioavailability data and to efficiently translate this towards the clinic, the following criteria should be considered: According to the EURL ECVAM principle on test validation, robust protocols to reproducibly generate hiPSC-derived BCECs and resultant BBB models should be provided, describing the purpose of the test method, especially specifying the test system, the readout of the method, defined endpoints, the derivation and expression of results, acceptance criteria, the interpretation of the results and the implementation of adequate controls. Secondly, within-laboratory variability of the assay over time as well as the transferability to another laboratory have to be examined. In addition, the between-laboratory reproducibility should be addressed using a group of at least three qualified laboratories/test facilities at different sites performing a blinded study (Hartung et al., 2004). Advices in good cell culture practice for human primary and stem cell-derived models should be complied to increase the reproducibility of the assay and to ensure high-quality control standards (Pamies et al., 2018). By comparing the obtained results with a reference method, as for example the expression profile of cell-specific markers and transporters to human brain tissue biopsies or freshly isolated BMECs, the predictiveness of the test system can be evaluated. Furthermore, the transport results have to be compared to established *in vivo* and *in vitro* assays (e.g., PAMPA, Caco-2, *in vivo* studies of analyze brain penetration) to determine the robustness of the developed *in vitro* assay. For validation of transport studies a panel of well-defined reference substances, including BBB relevant drugs, small molecules, and biopharmaceuticals of different substance classes, covering the whole spectrum of permeability rates from slow to fast permeating substances, also including substrates of active transporters, should be used. If the predictive power of the hiPSC-derived BBB models should be compared to different species, it is advisable to investigate similar transport targets and processes, but also to include *in vivo* as well as *in vitro* models of the other species in order to exclude *in vitro* model set-up dependent effects. In order to increase the successful development of neurotherapeutics, particular attention has to be focused on the identification of disease-relevant transport targets and mechanisms, taking into account the relevant cellular populations based on hiPSC-derived disease models and variations in gene expression depending on age, sex, or disease stage. To advance the exchange of improved knowledge and to make data available, for example by installation of a online database, for academia as well as for industry, exchange of permeability data from *in vitro* and *in vivo* studies, protocols of standardized test procedures and drug reference sets might lead to an improved data quality for proper comparison of the results obtained by different researchers. This could enable faster validation and approval processes, as well as an increased predictive power of transport studies performed by means of *in vitro* models including an increased correlation to *in vivo* data.

FUTURE TRENDS IN DRUG DEVELOPMENT APPLYING HIPSC-DERIVED BBB MODELS

Monolayer models of the human BBB using transwells as a basis are most frequently used to study signaling pathways, transporter kinetics, binding affinities, or to characterize leads. To fully simulate the BBB integrity and complexity, including cell-cell communications and cell-matrix interactions more complex models are required (Bagchi et al., 2019). The interaction between different brain cell types, directly or indirectly via secreted soluble factors, as well as dynamic shear stress increase for example the expression of EC transporters, as well as TJs and promote cellular polarity. Implementing these factors might be decisive in the future development of e.g. personalized and disease-relevant BBB models.

Precision Medicine

Precision medicine (PM), also known as personalized medicine, is a novel approach of medical treatment taking into account the

individual characteristics of each patient. The approach relies on tailored therapeutic strategies based on identifying and understanding a person's unique molecular and genetic profile and vulnerability to certain diseases. Compared to the traditional approach ("one size fits all"), PM takes into account a patient's genetic and epigenetic risk factors or other biomarkers in addition to clinical information and can increase the chance to predict the most effective and safe medical treatment. Individual BBB properties recently became more and more relevant in this context (Vatine et al., 2019).

Patients suffering from AD and PD show altered BBB functionality at the beginning of the disease and a BBB breakdown in later stages, which is accompanied with neuronal dysfunction, loss of neuronal connectivity, and neurodegeneration (Bowman et al., 2007; Gray & Woulfe, 2015). PM taking into account patient-specific BBB properties could improve therapeutic success for patients suffering from neurodegenerative disorders like AD or PD because alterations of the BBB accompany disease progression (Sweeney et al., 2018). However, modifying BBB functions for treating AD still remains challenging for different treatment strategies including antibody-based approaches, increasing brain clearance of Aβ or antagonize aggregation (Panza, Lozupone, Logroscino, & Imbimbo, 2019). PM focuses on the analysis of genetic mutations of patients to identify suitable drug targets for therapy or risk assessment including the prediction of negative side-effects and the exclusion of non-responders. In AD, this may include considering the genotypes of APOE and ACE (both expressed in BCECs) as recently reported for the treatment with ACE inhibitors (de Oliveira, Chen, Smith, & Bertolucci, 2018). Moreover, modulation of calcineurin-nuclear factor of activated T cell (NFAT) signaling could be another therapeutic option in APOE4-mediated CAA and AD (Blanchard et al., 2020).

HiPSC-based therapies for treating age-related macular degeneration have been successfully conducted (Zarbin, Sugino, & Townes-Anderson, 2019) and cell replacement therapies for human brain cells are currently being tested in clinical trials. A fascinating approach in PM is the transplantation of autografts into the CNS aiming for a curative approach of neurological disease (Barker, Parmar, Studer, & Takahashi, 2017), in particular in PD. The increasing number of clinical trials involving therapies based on hiPSC-derived cells shows the general applicability of autologous clinical-grade stem cells for cell replacement therapies. Additionally, gene therapy is discussed as a promising technology to restore functionality in diseased tissue. Recently, primary rodent BCECs were successfully transduced targeting the lysosomal cholesterol storage disease Niemann Pick type C2 (Hede et al., 2019). To target AD- and PD-related BBB symptoms, there is a need to first understand the consequence of disease-associated genetic variants. Then, patient-specific hiPSCs could be genetically modified to produce healthy BBB tissue for autologous transplantation, although current technologies and associated costs are still prohibitive.

Disease Models Mimicking BBB Alterations

The major promises of disease models are to improve our understanding of pathomechanisms and associated biomarkers. This could be achieved by hiPSCs since one key limitation in drug development for disorders of the CNS is the inaccessibility of (intact) brain tissue. Accordingly, there is a lack of adequate models with high predictive value.

The application of disease models with patient- and disease-specific hiPSC-derived cell types opens up the possibility (i) to develop more effective personalized therapies, (ii) to characterize novel drugs, (iii) to characterize disease-associated mutations and resulting malformations that occur in embryogenesis, childhood, adulthood or old age, (iv) to develop disease- and/or tissue-specific disease models *in vitro*, and (v) to mimic disease-associated mutations or to introduce artificial mutations (e.g. gene knockout) by gene editing (e.g. CRISPR/cas technology).

Diseases of the CNS are often multifactorial including a variety of environmental and genetic risk factors. Genetic risk factors are DNA variations including copy number variations (CNVs), single-nucleotide polymorphisms (SNPs), and functional homozygous mutations. DNA variations influence onset, progression, and treatment of neurodevelopmental and neurodegenerative diseases as well. In this context, DNA variations in genes regulating BBB functionality may not only have an impact on disease onset and progression, but may also play a role for disease treatment. Accordingly, the APOE status is detected as a biomarker in many preclinical studies. Disease models provide a powerful tool to interpret findings from

genetic studies including the validation of AD risk variants such as loss-of-function mutation found in ABCA7 representing a transporter expressed on BCECs (Sims et al., 2017). To investigate BBB dysfunction depending on individual genetic differences, individual cellular models need to be established according to patient-specific genetic profiles. HiPSC-derived models are ideal candidates for disease recapitulation enabling the analysis of molecular and cellular pathways in order to nominate suitable molecular targets addressing a dysfunctional BBB. Patient-derived hiPSCs can be used to investigate the underlying genetically driven pathomechanisms and pathways in BBB dysfunction, since they most likely maintain patient-specific genetic mutations also after transduction and differentiation. Genetic studies have already been conducted for over 20 years and the first familial AD-correlated mutation on chromosome 21 was already suggested in 1987 (St George-Hyslop et al., 1987). Later on, highly multiplexed genotyping sped up the identification of genetic causes of neurological disorders in patients (Sims et al., 2017). Following results from these genome-wide association studies, numerous projects aimed for the identification of disease-specific mutations and the resulting dysregulation or functional impairment of proteins in neurodegenerative disorders. The number of genetic risk factors associated with BBB dysfunction in neurodegenerative disorders is constantly growing due to the growing number of cohort studies and the enlargement of existing cohorts (Savage et al., 2018). The technological advancement in multi-omics technologies will further accelerate and enlarge such studies. Meta-analysis and epidemiological studies will support these developments. These data will help to fill gaps in our knowledge regarding of the contribution of altered BBB functionality in AD, PD, and other diseases. However, identifying the causal relationship between specific mutations and pathophysiological changes of the BBB and especially BCECs has only been addressed scarcely.

Cellular two-dimensional and organotypic three-dimensional hiPSC-derived *in vitro* models hold a great promise for the functional characterization of DNA variations necessary to further advance the field of translational medicine aiming at the transfer of insight from neuroscience research to clinical application. Patient-specific hiPSC-derived cells can also mimic aspects of BBB associated glial cell types including microglia which are key modulators of CNS disease (Ormel et al., 2018). Complex BBB models are suggested to be more predictive for disease modeling and toxicity screenings (Qian et al., 2018). Advanced co-culture CNS models composed of patient-specific hiPSC-derived cell types allow for the analysis of cell-cell communication and provide deeper insights into complex brain physiology (Raja et al., 2016; Smits et al., 2019). Thus, the impact of disease-associated mutations on BBB integrity in AD and PD patients can be studied in different cell types of the NVU. It is important to mention that mutations associated with AD occur in genes that are expressed by a variety of different cell types including BCECs, PCs, ACs, and neurons (Anttila et al., 2013; Blanchard et al., 2020; Challen et al., 2011). Therefore, in complex diseases such as AD and PD, however, multifactorial etiologies have to be considered, thus just cultivating AD hiPSC-derived BCECs might be not sufficient to mimic the disease phenotype and factors such as additional cell types of the NVU, inflammatory cytokines, growth medium adaptations or further disease-relevant stimuli have to be considered for *in vitro* model optimization.

Personalized *in vitro* cell models of the BBB, generated from patient-derived hiPSCs, could be used to validate drug candidates or to repurpose launched drugs for different indications. In preclinical testing, these models might have the potential to improve the success rate in clinical trials of CNS disorders affecting the BBB including AD and PD. Over 5000 clinical trials analyzing a variety of interventions for the treatment of neurodevelopmental diseases are currently listed in the United States National Institute of Health database *ClinicalTrials.gov*. Only 37 of them are related to the BBB. Application of preclinical hiPSC-derived BBB models to (i) improve the prediction of drug transport across the BBB to the target location, (ii) optimize efficient drug delivery, (iii) avoid potential risks, and (iv) eliminate unsuitable drug candidates early in the discovery/development phase could streamline this process and increase awareness for BBB-related aspects for CNS drugs.

Innovative Matrices and Three-Dimensional BBB *In Vitro* Models

In recent years, there has been a shift towards the development of more complex BBB models to better mimic brain functionality and physiology. To this end, the cellular

composition and extracellular milieu of the *in vitro* models was further improved. The ECM has a significant role in influencing cellular behavior, like differentiation, proliferation and cell attachment. As of now, single proteins are typically in use to increase specific cell behavior; however, this does not represent the complex *in vivo* extracellular microenvironment. Recent advances in biomaterial engineering has enabled the development from standard two-dimensional monolayer cell culture to three-dimensional approaches of CNS models. Cell-loaded hydrogels are in focus as an ECM for three-dimensional brain models, as it possesses many aspects of the natural ECM including stiffness, enzymatic degradability, and binding ligands for cell adhesion (Katt et al., 2018; Tibbitt & Anseth, 2009). Especially for neural stem cell engineering, the understanding and the role of ECM is of growing importance (Lam et al., 2019; Murphy, Haynes, Laslett, Cameron, & O'Brien, 2020; Yang et al., 2012), which is related to the increasing interest in the development of neural transplants for therapy of neurodegenerative diseases and stroke. Neuronal progenitor cells (NPCs) and glia cells can be differentiated from hiPSCs; however, there is a lack of efficient *in vitro* models that generate functional neuronal cells. Novel hydrogels seem to be able to provide promising ECMs for neural differentiation to matured and functional cells for e.g. stroke implants (Lam, Lowry, Carmichael, & Segura, 2014; Moshayedi et al., 2016), and are therefore a current focus for biomolecular engineered stem cell transplants (Nih et al., 2017). Tissue engineered scaffolds like decellularized porcine brain tissue may also be utilized to build up a three-dimensional model of the CNS, since it consists of brain-specific matrix components, like glycosaminoglycans, collagen I, collagen III, collagen IV, collagen V, collagen VI, perlecan, as well as laminin (DeQuach, Yuan, Goldstein, & Christman, 2011). This porcine brain matrix can be used as a platform for hiPSC-derived cells of the NVU to build up a three-dimensional brain model and to support vascularization. Alternatively, it can be applied as coating reagent to enhance cell adhesion or to generate artificial hydrogel-based nanofibrous scaffolds (DeQuach et al., 2011). Despite of the great potential of such natural brain-specific tissue scaffolds, they are still from animal origin, which might pose a problem of species specificity. In this regard, it was shown that the usage of human-specific laminin led to better astroglia differentiation compared to the murine protein (Delsing, Kallur, Zetterberg, Hicks, & Synnergren, 2019), also the integrity of hiPSC-derived BCECs was even improved by human laminin compared to BCECs differentiated on (mouse-derived) Matrigel (Aoki, Yamashita, Hashita, Iwao, & Matsunaga, 2020). Cho et al. established a promising electrospun nanofibrous scaffold based on human brain ECM derived from decellularized human brain tissue to enhance the differentiation of hiPSCs into functional, myelin-expressing oligodendrocytes (Cho et al., 2019). Similar approaches might work also for the establishment of improved BBB models. To avoid possible ethical issues due to the usage of human tissue, an increasing number of studies focus on advanced self-assembling cell types to simulate *in vivo* three-dimensional tissue architecture, such as BBB spheroids, neuronal organoids and mini-brains. These approaches avoid the application of additional ECM constituents (Campisi et al., 2018; Lancaster et al., 2013; Pasca et al., 2015; Urich et al., 2013). The major advantage of these three-dimensional models over two-dimensional cocultures is the direct cell-cell contact, which is crucial for the activity of key signaling pathways. Furthermore, hiPSC-derived three-dimensional *in vitro* BBB models allow to replicate *in vivo* architecture and offer an exciting possibility to study species-specific processes in health and disease in order to develop novel assays suitable for drug discovery (Cho et al., 2017; Nzou et al., 2018).

Dynamic Flow and Microfluidic Culture Systems

BCECs, lining the lumen of capillaries and microvessels, are under constant shear stress generated by blood flow. Although it is known that the application of shear stress affects BCEC characteristics, the majority of the BBB models are represented as static models, (Cucullo, Hossain, Puvenna, Marchi, & Janigro, 2011; DeStefano, Xu, Williams, Yimam, & Searson, 2017; Faley et al., 2019; Garcia-Polite et al., 2017; Wang et al., 2017). Vascular ECs under laminar flow show elongation, polarization, and alignment in the direction of the flow (Ballermann, Dardik, Eng, & Liu, 1998; Tkachenko et al., 2013). The effects of shear stress on BCECs seem to be dependent on the BCECs origin and their tightness status. Especially, hiPSC-derived BCECs showed increased resistance to shear stress mediated elongation in comparison to immortalized BCEC lines with lower basic

tightness (DeStefano et al., 2017; Reinitz, DeStefano, Ye, Wong, & Searson, 2015; Ye et al., 2014). Despite the contradicting data on the role of shear stress and continuous supply of fresh media in different models, the influence of flow is not negligible for BCECs, but it is currently unclear how decisive this factor is for the generation of functional barriers. Different kinds of dynamic BBB models including medium flow are in use to mimic the *in vivo* situation more closer. One example is the application of spinning bioreactors to adapt and maintain mini-brains or spheroids in order to ensure an optimal nutrient distribution within the culture vessel (Qian et al., 2018; Qian et al., 2016; Yan, Song, Madinya, Ma, & Li, 2018). Thereby, the generation of large scale hiPSC-derived BBB spheroid models seems to be feasible and could be used to provide a platform for modeling human brain development and disease (Miranda et al., 2015; Qian et al., 2018; Qian et al., 2016). Engineered microvessels and chip models are another example to apply physiological sheer stress on BCECs (de Graaf et al., 2019). Hydrogel casts with a few hundred-micron range thickness allow the observation of cellular behavior in a more biomimetic environment including fabricated channels supporting fluid flow within the gel (Bertassoni et al., 2014; Lee et al., 2020; Miller et al., 2012). In engineered microfluidic models, such as three-dimensional gelatin/hydrogel channels or so-called brains-on-a-chip, hiPSC-derived BCECs represented a much more stable barrier function and a significant barrier tightness compared to static controls (Faley et al., 2019; Katt et al., 2018; Wang et al., 2017). Moreover, the addition of shear stress on hiPSC-derived BBB models increased the longevity and highlighted their potential application for studies lasting up to three weeks (Faley et al., 2019). The future developments in advanced microfluidics and their application will show (if they are able to hold their promise to recapitulate *in vivo* BBB physiology). To make them ready for industrial use, additional distinct efforts are necessary.

CONCLUSION

The decisive criterion for BBB model selection is the purpose of the study to be conducted (Bagchi et al., 2019). One could follow the credo: As easy as possible, as complex as necessary. A comparison of the currently available drug permeability datasets obtained from BBB *in vitro* models is difficult due to the diversity of the model setups applied, the characterization of variable sets of compounds targeting a variety of transport mechanisms, and the differences of the applied BCECs (Stanimirovic, Bani-Yaghoub, Perkins, & Haqqani, 2015). Moreover, complex dynamic *in vivo* processes, such as drug pharmacokinetics, brain exposure, distribution, elimination, target engagement, and also efficacy, cannot be fully replaced by *in vitro* approaches (Stanimirovic et al., 2015). We see human BBB models as an initial drug screening platform to reduce overall costs in drug development (Aday et al., 2016). Specific questions like the capacity of paracellular diffusion, directional transport across the BBB, rates of transcellular transport, efflux of substances, carrier-mediated transport, receptor-mediated transcytosis, drug metabolism/degradation by BCECs, immune cell interactions with the BBB, screening for potential BBB Trojan horses, species differences/selectivity, as well as cell-based toxicity can be answered by this technology (Stanimirovic et al., 2015). Therefore, the development, validation, and standardization of BBB models is key. HiPSC-derived BBB *in vitro* models showed high correlation to the human *in vivo* situation, but much effort has to be invested on validating these *in vitro* models in order to achieve their broad acceptance, also in industry. Additional aspects requiring critical evaluation are hiPSC-specific model variations, the long-term stability of these BBB models (Aday et al., 2016), and the integration of age-related phenotypes, since BBB models derived from pluripotent stem cells mostly represent young individuals (Lauschke, Frederiksen, & Hall, 2017). Recently, a variety of improved and complex hiPSC-derived BBB models was developed, considering relevant factors such as direct cell-cell contacts, modulation of barrier properties by specified ECM components or flow-induced shear stress, and three-dimensional structures. Nevertheless, due to cost efficiency, handling simplicity, options for automatization, time efficiency for reaching steady-state and comparative reasons, transwell-based BBB models currently seem to be the models with the most potential to be integrated into industrial testing regimes. Monolayer- or isogenic co-culture *in vitro* models of the NVU are considered the gold standard in the field of BBB research and should be incorporated into CNS drug discovery and development programs after evaluation and optimization of the desired features.

The combination of standardized *in vitro* and *in vivo* approaches will improve the translation of data and hopefully clinical success of drug development targeting CNS disease by designing safer and more efficient drug delivery systems (Bicker et al., 2014; Stanimirovic et al., 2015).

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KEY REFERENCES

Abbott & Friedman, 2012. See above.

Gave a fundamental overview and introduction to understand the BBB in health and disease.

Lippmann et al., 2012. See above.

Published for the first time a differentiation method to generate purified BCECs from hiPSCs. These hiPSC-derived BCECs are characterized by in vivo-like properties. This work pioneered the application of patient-specific hiPSC-derived BBB models in research.

Urich et al., 2013. See above.

Investigated a three-dimensional multicellular spheroid model of the BBB, presenting for the first time a spontaneous and complex self-organization of multiple BBB cell types. Most importantly, this process was not dependent on artificial scaffolds, supporting the hypothesis that the formation and cellular architecture of the BBB is intrinsically programmed within the specific cell types.

Patel, Page, & Al-Ahmad, 2017. See above.

Established an isogenic complex BBB model derived from patient-specific hiPSCs and investigated the impact of inter-individual genetic variations on the yield and phenotype of isogenic BBB models. Differences in differentiation capacity, maturation, and barrier properties between the models derived from different hiPSC lines supported the impact of individual polymorphisms in stem cell research and for precision medicine.

Prieto et al., 2004. See above.

Within the report of the forty-ninth workshop organized by the EURL ECVAM this key reference gave fundamental recommendations on the application and validation of BBB in vitro models in toxicology screening strategies.

Reichel, 2006. See above.

Reviewed the role of BBB studies for the pharmaceutical industry in preclinical drug discovery and development. Furthermore, the specific requirements of the pharmaceutical industry for the development of effective and safe new CNS medicines were summarized.