Blood brain barrier: An overview on strategies in drug delivery, realistic *in vitro* modeling and *in vivo* live tracking

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Keywords: blood brain barrier (BBB), brain on a chip, central nervous system, nanocarrier, strategies, tight junction

Abbreviations: BBB, Blood brain barrier; SPECT, Single photon emission tomography; FUS, Focused ultrasound; LITT, Laser interstitial thermotherapy; NTIRE, Nonthermal irreversible electroporation; HIV, Human immunodeficiency virus; oxLDL, Oxidized low density lipoprotein; AD, Alzheimer's disease; PD, Parkinson's disease; NIRF, Near infrared fluorescence; MRI, Magnetic resonance imaging; PET, Positron emission tomography; CT, Computerized tomography; CENI, Contrast enhanced nuclear imaging; CE-MRI, Contrast-enhanced MRI; CA, Contrast agents; CNS, Central nervous system; VEGF, Vascular endothelial cell growth factor; Shh, Sonic Hedgehog; RA, Retinoic acid; TGF- β , Transforming growth factor β ; BMP, Bone morphogenetic proteins; Ang, Angiopoietins; Sema, Semaphorins; RALDH, Retinaldehyde dehydrogenase; BCSFB, Blood–cerebrospinal fluid barrier; ISF, Interstitial fluid; TJ, Tight junction; TWEAK, TNF-like weak inducer of apoptosis; SHRSP, Spontaneously hypertensive stroke-prone; MMPs, Matrix metalloproteinases; GBS, Group B *Streptococcus*; LPA, Lysophosphatidic acid; PEG, Polyethylene glycol; PAMAM, Polyamidoamine dendrimers; NP, Nanoparticle; PLGA, Poly(lactic-co-glycolic acid); MBs, Microbubbles; MNP, Magnetic nanoparticles; RF, Radiofrequency field; RMT, Receptor-mediated transcytosis; AR, Adenosine receptor; CPPs, Cell-penetrating peptides; RVG, Rabies Virus Glycoprotein; CTX, Chlorotoxin; TNF, Tumor necrosis factor; SOD, Super oxide dismutase enzyme; WNV, West Nile virus; TEER, Trans-epithelial electrical resistance; RBEC, Rat brain endothelial cells; DOC, Deoxycholic acid; LSCI, Laser speckle contrast imaging; 18F-FDG, 18F-2-fluoro-2-deoxy-d-glucose; ECS, Extracellular space; IOI, Integrative optical imaging; NSCs, Neural stem cells; FTD, FeraTrack Direct; MCAO, Middle cerebral artery occlusion.

Blood brain barrier (BBB) is a group of astrocytes, neurons and endothelial cells, which makes restricted passage of various biological or chemical entities to the brain tissue. It gives protection to brain at one hand, but at the other hand it has very selective permeability for bio-actives and other foreign materials and is one of the major challenges for the drug delivery. Nanocarriers are promising to cross BBB utilizing alternative route of administration such as intranasal and intra-carotid drug delivery which bypasses BBB. In future more optimized drug delivery system can be achieved by compiling the best routes with the best carriers. Single photon emission tomography (SPECT) and different brain-on-a-chip *in vitro* models are being very reliable to study live *in vivo* tracking of BBB and its pathophysiology, respectively. In the current review we have tried to exploit mechanistically all these to understand and manage the various BBB disruptions in diseased condition along with crossing the hurdles occurring in drug or gene delivery across BBB.

Introduction

The BBB is a gatekeeper formed by the endothelial cells that line cerebral microvessels^{1–4}. In spite of moving para-cellularly through the junctions, as in most endothelia complex tight junctions between adjacent endothelial cells force most molecular queue to take a trans-cellular route across the blood brain barrier (BBB).^{5,6} Now, the embryonic BBB studies has boosted our understanding of the cellular and molecular mechanisms of BBB development. In the same way angiogenesis exploration, brain vascular development pathways e. g. Wnt or DR6/TROY (death receptor) pathways^{7–10} and genes important for angiogenesis such as norrin and Mfsd2a^{11,12} have been reported. Lipid-soluble drugs with <400 Da can go across the BBB but large and hydrophilic molecules can't. By temporary disruption of BBB therapeutic agents can be potentially administered to the brain by neurosurgery. Majorly employed neurosurgical BBB disruption methods are intra-arterial mannitol infusion (hyper-osmotic therapy), focused ultrasound (FUS), laser interstitial thermotherapy (LITT), and non-thermal irreversible electroporation (NTIRE).¹³ HIV viruses,¹⁴ angiotensin-II,¹⁵ hypoxia,^{16,17} increased circulating oxidized LDL (oxLDL)¹⁸ are also responsible for brain disruption in diseased conditions. Polymeric nanoparticle, lipid-based nanoparticle, liposome, micelle, dendrimer and carbon nanotube are very efficient nanocarriers to cross BBB in neurodegenerative disorders like Alzheimer disease (AD) and Parkinson disease (PD).¹⁹ Emphasizing on bypassing BBB by

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Submitted: 11/03/2015; Revised: 12/01/2015; Accepted: 12/04/2015
<http://dx.doi.org/10.1080/21688370.2015.1129476>

intranasal drug delivery is great area of interest nowadays,²⁰ along with osmotic opening of BBB by intracarotid infusion of hypertonic solutions (arabinose or mannitol)²¹ and pathogenic strategies exploiting chlorotoxin as ligand.²² Models mimicking the *in vivo* anatomic-physiological complexity of the BBB are also available, including triple co-culture (culture of brain endothelial cells in the presence of pericytes and astrocytes), dynamic, and micro-fluidic models.²³ Bobilya DJ illustrated BBB model for transport mechanism studies as a co-culture model with astrocytes and capillary endothelial cells from pig brain.²⁴ Non-invasive imaging is commonly done with near infrared fluorescence (NIRF), magnetic resonance imaging (MRI), positron emission tomography (PET), single photon emission tomography (SPECT) and X-ray computerized tomography (CT). Contrast enhanced nuclear imaging (CENI) can quantitatively assess cellular processes. Along with SPECT and CT to evaluate BBB integrity, MRI is the mostly used technique. Contrast-enhanced MRI (CE-MRI) sequences are used for BBB imaging in human, where gadolinium (Gd)-based contrast agents (CA) that do not cross the intact BBB are used.²⁵⁻³⁰

In the present review we have attempted to discuss and compile the implications of BBB in the efficient drug delivery by crossing or bypassing BBB using different *in vitro* models along with their exact *in vivo* imaging by advanced technology. The manuscript also focuses on the classical aspects of the nanotechnological tools and their role in the brain delivery either bypassing or disrupting BBB. In the recent years these nanocarriers has played a key role in the drug delivery particularly in the controlled and sustained drug delivery to brain and other body organs.

Recent Advances in Understanding the Development, Structure and Functions of the BBB

Development of the blood–brain barrier

The initial communications of the embryonic endothelium with neural cells triggers the BBB sprouting.³¹ The BBB matures during foetal life and is well formed by birth.³²⁻³⁷ The development of the vascular endothelium is now believed to be induced by neuro-epithelial signaling through Wnt/ β -catenin pathway to induce a CNS-specific vascular system and BBB specialization.^{7,9,38} Early feature in BBB development is the formation of tight junctions. In humans, a brain of a 14 week fetus express occluding and claudin-5 in the capillary endothelium with the same distribution at cell margins similar to an adult.³⁹ Human post-mortem studies of perinatal deaths and stillborn fetuses from approximately 12 weeks gestation have revealed that a barrier exist from at least the beginning of the second trimester, as in adult human.⁴⁰ Astrocytes have a key role in regulating the tightness of the BBB.^{41,42} Vascular endothelial cell growth factor (VEGF) along with its endothelial tyrosine kinase receptors VEGFR1 (Flt-1) and VEGFR2 (Flk-1) have been evidenced for CNS angiogenesis. Heparin-binding domains provides different VEGFs the ability to attach to the extracellular matrix.⁴³ Extracellular VEGF gradients (Fig. 1) are recognized by

VEGFR2-expressing endothelial cells at the tip of the vascular sprouts which forms filopodial extensions and are therefore referred to as endothelial tip cells.⁴⁴ The development of BBB comprises 3 major phases. In the first phase development of the BBB begins with angiogenesis when endothelial progenitor cells invade the embryonic neuroectoderm (Fig. 1A). In the second phase basement membrane formation and limiting permeability phase of BBB development is occurred (Fig. 1B). In the third phase BBB gets mature and tight junctions are formed along with the required maintenance of BBB (Fig. 1C). Semaphoring/VEGF co-receptor neuropilin 1 (Nrp1), recognizes heparin-binding isoforms of VEGF.⁴⁵

Sonic hedgehog (Shh), Wnt/ β -catenin, retinoic acid (RA), transforming growth factor β (TGF- β), bone morphogenetic proteins (BMP), angiopoietins (Ang), and semaphorins (Sema) are the most important morphogens serving as signaling factors facilitating cell differentiation by providing positional information to developing tissues. Morphogens are also present in a gradient which determines a specific pattern of gene expression in that particular cell. Numerous morphogens which induces neurogenesis are also believed to involved in BBB development and maintenance.⁴⁶ GPR124 (G-protein coupled receptor) linked to VEGF and TGF- β signaling pathway have been found in brain vascular morphogenesis. Wnt/ β -catenin and GPR124 are involved in BBB formation beside of angiogenesis development through these similar pathways. Down regulation or absence of GLUT-1 expression in the CNS vasculature signifies induction of endothelial cell differentiation.⁴⁷ It is found that DR6/TNFRSF21 and TROY/TNFRSF19 as regulators of CNS-specific angiogenesis and observed that the attaining of these death receptors induced BBB leakage in both zebrafish and mice but GLUT-1 expression remains unaffected.¹⁰ VEGF mediated JNK activation *in vitro* and the Wnt/ β -catenin pathway are linked to death receptors. With a zebra fish model used to test involvement of several candidate genes it was found that DR6, TROY, and Spock2 acquiring revealed for vascular malformations as well as barrier defects. But in the absence of barrier leakage phenotypes *Adcyap1r1* and *Tspn5* genes have shown gross brain vascular malformations too. BBB formation is not affected by aberrant vascular morphogenesis. Surely, pathological glomeruloid tufts and hemorrhage in some of the mutants could also be found in a knock out for the neuropilin 1 gene.⁴⁸ Therefore, affecting the VEGF pathway associated with down regulation in GLUT-1 expression is believed. A down-regulation in tight junction proteins in the embryonic vasculature of genetic mouse models targeting the sonic hedgehog (SHH) pathway is reported.⁴⁹ It is observed that serum protein extravasations at E14.5 as an indication of barrier dysfunction, without any in the total number of blood vessels at E13.5. Specific barrier-genesis effect is also found with genetic extirpation of the *Norrin/Frizzled4* pathway.¹ Loss of BBB integrity is reported by tracer perturbations and leakage in the expression of BBB markers in certain regions of BBB.⁵⁰ At least in mouse models there seem to be a temporal separation. In its cortex, angiogenesis starts with vessel admittance into the neural tube around E9-E10 while barrier sealing properties within these vessels mature around E15-E16.^{1,12} The beginning of BBB

Figure 1. Major signaling pathways in BBB development.

transporters expression e.g. of GLUT-1 links with the induction of CNS angiogenesis. The cortex is still very active in angiogenesis. In the bandicoot, it is found that the developing vessels that grow into the brain have already a functional barrier.⁵¹

In humans fork head transcription factor, FOXF2 have been found for stroke perceptivity and is specifically expressed in pericytes of the brain and that FOXF2 (-/-) embryos develop intracranial hemorrhage, perivascular edema, thinning of the vascular basal lamina, an increase of luminal endothelial caveolae, and a leaky BBB.⁵² Retinaldehyde dehydrogenase (RALDH) is responsible for RA production and is expressed by radial glial cells in the developing human CNS. The release of RA by radial glial cells has also been shown by others, as well as the dependence of neural precursors on radial glia-derived RA as a differentiation factor.⁵³

Structure of the BBB

All organisms with a well-developed CNS have a BBB⁵⁴ which is formed from the endothelial cells that form the inner walls of the capillaries in brain and spinal cord. The combined surface area of these micro-vessels is between 150 and 200 cm² g⁻¹ tissue and the total area for exchange in the brain is between 12 and 18 m² for the average human adult.⁵⁵ The blood-cerebrospinal fluid barrier (BCSFB) constitutes the epithelial cells of the choroid plexus facing the cerebrospinal fluid. Across the choroid plexus epithelial cells the cerebrospinal fluid (CSF) is secreted into the ventricular system of the brain⁵⁶, while across the capillary endothelium of the BBB the interstitial fluid (ISF) and extracellular fluid (ECF), are partially secreted.^{54,57-59} The ISF to CSF contribute 10 to 60% in communication at various regions.^{60,61} In the apical membrane of the choroid plexus epithelium and abluminal membranes of the BBB endothelium there exists ionic and osmotic gradient generated by the Na⁺, K⁺-ATPase. ISF and CSF are secreted via this gradient. Volume flow and water movement are the outcomes from these incidents.⁵⁴ Avascular arachnoid epithelium

under dura completely encloses the CNS. Thus it isolates the extracellular fluids (ECF) of the central nervous system from the rest of the body.⁶² Due to the limitations of arachnoid like very small surface area and devoid of any vascularity. It hasn't any great role in BBB function although it provides a matrix to BBB. (Fig. 2).⁶³ All these constitutively make 3 boundary walls for BBB in the form of physical barrier, transport barrier and metabolic barrier aiding tight junctions, chemical flux and enzymatic metabolisms respectively.⁶²

The major dimensions of the BBB are as follows: brain capillary length = 650 km; brain capillary volume = 1 mL; surface area of the luminal capillary $\approx 12 \text{ m}^2 \equiv 100\text{--}240 \text{ cm}^2/\text{g brain}$; thickness of the BBB = 200–500 nm; luminal diameter of brain capillaries $\approx 7 \mu\text{m}$ in humans and $4 \mu\text{m}$ in rats; mean distance between 2 capillaries $\approx 40 \mu\text{m}$; transit time of blood $\approx 5 \text{ s}$; capillary volume = $11 \mu\text{L/g brain}$; interstitial fluid (ISF) $\approx 20\%$ of the brain parenchyma;^{65,66} ISF flow toward the CSF in rats $\approx 0.15\text{--}0.29 \mu\text{L/min/g}$;⁵⁴ CSF volume in humans $\approx 160 \text{ mL}$; CSF volume in rats $\approx 250 \mu\text{L}$ in rats; rate of CSF secretion in humans $\approx 350 \mu\text{L/min}$; rate of CSF secretion in rats $\approx 2.1 \mu\text{L/min}$.^{67,68}

Cell mediated functions of the BBB

The BBB performs 3 major functions: it curbs free transport between the blood and the brain, essential nutrients are supplied to the brain through it, and it aid to flow out any harmful or toxic waste or foreign substances. The brain endothelial cells which form the BBB expresses transport proteins. These are mostly separated within either the luminal or abluminal surfaces which mainly give expressions for the transport of peptides, proteins and neurotransmitter metabolizing enzymes (Table 1). The two major mechanisms of transport across BBB (Fig. 3) are carrier mediated transport (Fig. 3A) and receptor mediated transcytosis (Fig. 3B).^{12,43} The BBB functions through various cells are summarised as follows:

Astrocytes

Astrocytes provide the cellular colligate to the neurons and are derived from ependymoglia of the developing neural tube, and retain some features of their original apical–basal polarity, together with more specific polarization of function in relation to particular cell–cell associations of the adult.

- A. Increased integrity of the BBB.⁶⁹
- B. Tight junction (TJ) expression and TJ complex formation and maturation, expression and localization of brain EC transporters, and specialized enzyme systems have been shown to be up-regulated under astrocyte influence.⁵
- C. Transforming growth factor- β (TGF β) secreted by astrocytes has been shown to mediate the regulation of tissue plasminogen activator and the anticoagulant thrombomodulin.⁴¹
- D. Sonic hedgehog (Shh), a member of the Hh pathway, was shown to be produced and secreted by perivascular astrocytes in the human and mouse adult brain and that microvascular brain ECs expressed the receptors and the intracellular machinery to respond to Hh ligands. Pharmacological

neutralization of Hh receptors or genetic deletion of Hh receptors lead to enhanced permeability of the BBB and loosening of the TJs.⁷⁰

Pericytes

- A. Perivascular pericytes are known to release growth factors and angiogenic molecules which are able to regulate microvascular permeability and angiogenesis.⁶⁹
- B. Contribute to the stability of microvessels and cover a large part of the abluminal brain EC surface, for BBB permeability.^{5,62}
- C. Pericytes contracting and relaxing in a regulated manner regulates blood flow in CNS capillaries.⁶⁶

Neurons

- A. Indirect regulation of blood flow.⁶⁶
- B. Directly innervate brain EC or brain EC-associated astrocytes functioning as a liaison for neuronal–endothelial coupling.⁵
- C. Directly influence BBB permeability, through direct innervations of brain EC.²⁴

The Mechanisms Underlying Disruption of BBB

The BBB is disrupted in several acute and chronic neurological disorders. These include epilepsy, Alzheimer disease, Parkinson disease, multiple sclerosis, dementia⁶⁸ cerebral ischemia,⁷¹ traumatic brain injury, stroke, neuro-myelitisoptica, human immunodeficiency virus (HIV) encephalopathy, glioblastoma, bacterial meningitis,⁷² and pain⁷³ (selected conditions are enlisted in Table 2). The BBB also appears to be impaired in some non-CNS diseases such as rheumatoid arthritis,⁷⁴ diabetes,⁷⁵ liver failure,⁷³ eclampsia,⁷⁶ hypertension,⁷⁷ and atherosclerosis.⁷⁸ The loosening of the tight junctions, the downregulation of the tight junction proteins, and the degradation of the capillary basement membrane are the mode of disruption of BBB.²⁵ BBB breakdown and increased capillary permeability aid the passage of inflammatory cells into the brain tissues and the exudation of serum proteins or other neurotoxic plasma constituents. Activated of astrocytes and microglia results in edema, deposition of toxic substances in the brains IF, oxidative stress, and impaired homeostasis, neuro-inflammation, remodelling of vasculature, and emended synaptic plasticity. BBB disruption may act as the initiating trigger of many neurological disorders.⁷⁹ Altered expression of transporter proteins are also the consequences with BBB disruption in diseased conditions.

Some of the other physiological and diseased conditions are also associated with the BBB disruption.^{17,25,80,81} Vitamin D prevents hypoxia or reoxygenation-induced BBB disruption via vitamin D receptor-mediated NF- κ B signaling pathways. It is hypothesized that 1, 25(OH) 2D3 blocks NF- κ B-mediated hypoxia-induced BBB disruption and cerebral endothelial cell death by binding to the VDR, and this beneficial effect is blocked by P5P.¹⁶

In human brain microvascular endothelial cells *in vitro*, TNF-like weak inducer of apoptosis (TWEAK) decreases tight

Figure 2. Structure of BBB.

junction ZO-1 expression and increases the permeability of monolayer cell cultures and administration of Fc-TWEAK intravenously directly increased the leakage of a tracer (dextran-FITC) into brain tissue. MRL/lpr Fn14KO mice displayed reduced antibody (IgG) and complement (C3, C6, and C4a) deposition in the brain.⁸² In epilepsy albumin can be taken up by the astrocytes via transforming growth factor β receptors proceeding with down regulation of aquaporin 4 channels in these astrocytes resulting in reduced buffering of extracellular potassium, which aids N-methyl-D-aspartate-receptor-mediated (NMDA) neuronal hyper excitability and finally convulsion.⁸³

onstrated that lysophosphatidic acid (LPA) present in the blood delivered in intra-cerebroventricular hemorrhages mediates the disruption of the neuroepithelium.⁸⁵

Potential Gateways for Drug Delivery to the Brain

One of the major hurdles for the effective treatment of CNS disorders is the presence of the tightly regulated BBB, and its selective permeability which prevents most of the bioactive molecules from entering to brain. Therefore methods to bypass or

Increased oxidative stress occurs in spontaneously hypertensive stroke-prone rats (SHRSP), and play a great role in BBB disruption. Reduction in oxidative stress can be achieved by hydrogen in various diseases. Recent studies investigated for long-term hydrogen treatment improves neurological function outcome in the SHRSP model in the oxidative stress and the activity of matrix metalloproteinases (MMPs).⁷²

Group B *Streptococcus* (GBS) is the leading cause of neonatal meningitis. Bacteria-independent Snail1 expression aids tight junction disruption, which allows BBB permeability for bacteria. GBS induction of Snail1 expression depends on the ERK1/2/MAPK signaling cascade and bacterial cell wall components. Over expression of a dominant-negative Snail1 homolog in zebrafish elevated transcription of tight junction protein-encoding genes and increased zebrafish survival in response to GBS challenge. It is to believe that Snail1-dependent mechanism of BBB disruption and penetration is concurred by meningeal pathogens.⁸⁴ Impaired neurogenesis and corticogenesis results in the disruption of the germinal ventricular and subventricular zones. Spina bifida aperta and hydrocephalus, N-cadherin cell junctions are abnormally located in the cells of the ventricular zone of ventricle walls including the aqueduct in the fetuses causes BBB disruption. In human ependymal lining defects are implicated in the origin of hydrocephalus and in the eradication of the aqueduct. Recent evidence dem-

Table 1. Functions of BBB

Aspects	Functions
Ion regulation	By a combination of specific ion channels and transporters keeps the ionic composition optimal for synaptic signaling function. helps to keep the central and peripheral transmitter pools separate, minimizing 'crosstalk'
Neurotransmitters	
Macromolecules	
	Factor Xa is present in the brain, which converts prothrombin to thrombin, and the thrombin receptor PAR1 is widely expressed in the CNS. Similarly tissue plasminogen activator is present in central nervous tissues and converts plasminogen to plasmin. Thrombin and plasmin if present in brain ISF can initiate cascades resulting in seizures, glial activation, glial cell division and scarring, and cell death.
Neurotoxins	As a protective barrier which shields the CNS from neurotoxic substances circulating in the blood via ABC energy-dependent efflux transporters (ATP-binding cassette transporters) which actively pump endogenous metabolites or proteins, or xenobiotics ingested in the diet or otherwise acquired from the environment out of the brain.

cross the BBB and to deliver therapeutics into the brain are currently being investigated. The various strategies employed currently to cross BBB are enlisted in Table 3.

Strategies to cross the BBB

Nanotechnology

Nanotechnology has gave numerous options in the delivery of bio-actives has benefited the formulation delivery in several modes i.e. reduced side effects, targeting, improved patient compliance etc. Polymeric NPs are efficient nanocarriers for CNS drug delivery because of their drug encapsulation property which protects the drug from first pass metabolism, and in active constituent delivery across the BBB without damaging the BBB.⁸⁶ Solid lipid nanoparticles (SLNs), measuring 33–63 nm loaded with the antioxidant agent idebenone, have been shown to cross an *in vitro* model of the BBB via a transcellular pathway.⁸⁷ Liposomes with ApoE derived peptides facilitates cellular uptake and drug transport across a model of the BBB.⁸⁸ A nanomedicine for the delivery of drugs across the BBB has been prepared from chitosan NPs with an incorporated peptide. The surface was modified with polyethylene glycol (PEG) to enhance the plasma residence time by preventing NP capture by the reticuloendothelial system.⁸⁹ Techniques employing dendrimers are potentially useful for the systemic administration of drugs targeting in Alzheimer disease.⁹⁰ A pH sensitive dual targeting drug carrier (G4DOXPEGTf-tamoxifen) has

been synthesized with transferrin (Tf) conjugated on the exterior and tamoxifen in the interior of IV generation PAMAM dendrimers for enhancing the BBB transportation and improving drug accumulation in glioma cells.⁹¹ Nanogel PEG carriers, have been tested for brain delivery of activated nucleoside reverse transcriptase inhibitors for HIV1 into monocyte derived macrophages, which act as reservoirs for the virus.⁹² PLGA–PEG–PLGA triblock loaded loperamide has been formulated for drug delivery across the BBB.⁹³

Figure 3. Mechanism of transport across BBB.

Table 2. Different pathophysiological condition and BBB disruption

Conditions	Conditions Changes in BBB properties
Alzheimer	BBB leakage ↓ Regional CBF ECE2 activation ↓ P-gp expression LRP-1 and RAGE induced amyloid deposits Up-regulation of ABCG2
Parkinson	↓ TJ proteins ↓ mRNA coding for P-gp ↑ MRP2 expression
Epilepsy	↓ TJ proteins, TJ opening Albumin, IgG, and leukocyte extravasation ↓ regional CBF in temporal lobe epilepsy ↑ P-gp, MRP1, MRP2, and BCRP expression Link between ABCG2 polymorphism and pharmacoresistance
Multiple sclerosis	↑ CBF and PS ↓ TJ proteins Leakage of the BBB and inflammatory cell infiltration ↓ P-gp, MRP-1, and MRP-2 expression
Schizophrenia	Albumin and IgG extravasation
Depression	Albumin extravasation
Aging	↓ regional CBF Albumin extravasation ↓ P-gp expression

Hyperthermia techniques

Hyperthermia is a therapeutic procedure using increased temperature between 41 to 43°C to change the functionality of cellular structures in body tissues which kills the infected cells by leaving normal cells unaffected.⁹⁴⁻⁹⁶

Focused ultrasound (FUS) concentrate acoustic energy into a focal spot to produce selective disruption and increased permeability of the BBB. The technique also complies with the existing approved drugs. Microbubbles (MBs) incorporated into FUS enhance the FUS effects to the blood vessel walls with minimal damage to surrounding brain tissue.⁹⁷ Electrohyperthermia due to its higher conductivity and higher permittivity selectively affects the diseased tissues. Electromagnetic waves produced from radiofrequency or microwaves have been reported for hyperthermia to increase the permeability of the BBB *in vivo*. Radiofrequency for glioma treatment in combination with chemotherapy and radiotherapy is also reported recently.⁹⁸ An alternate method for hyperthermia is laser which is used to induce a selective region BBB disruption with the help of Nd:YAG laser pulse.⁹⁹ The delivery of large molecules using a near-infrared ultrashort pulsed laser are reported for BBB permeability induction.¹⁰⁰ Magnetic hyperthermia BBB permeability is also exploited for delivering bioactive compounds via heat generated from magnetic heating of MNPs. Low radiofrequency (RF) field magnetically excite MNPs to release energy in the form of heat to their surroundings through a mechanism called Néel relaxation. The technique is employed for treatment and diagnosis of disease.¹⁰¹

Receptor-mediated transport

Receptor-mediated transport (RMT) exploits the vesicular trafficking machinery of brain ECs to deliver transferrin, insulin, lectin, and lipoproteins and related proteins to the brain. It

follows 4 major steps: receptor-ligand binding on apical plasma membrane, endocytosis via membrane invagination and intracellular vesicle formation containing receptor-ligand complexes, routing of this intracellular vesicle via cell's vesicular and endolysosomal trafficking machinery to their respective destination, and in last step transcytosis and exocytosis occurs, which release constituents of vesicle into the brain parenchyma. It also involves transport of a wide range of endogenous proteins from uniform proteins like transferrin to large heterogeneous molecules like lipoproteins.¹⁰²⁻¹⁰⁵ BBB permeability can be upregulated by activating A2A adenosine receptor (AR), which temporarily increases intercellular spaces between the brain capillary endothelial cells.¹⁰⁶

Cell-penetrating peptides

Cell-penetrating peptides (CPPs), also known as protein transduction domains or membrane translocation sequences, have shown a reliable mechanism for passive delivery of biologically active molecules into cells. CPPs are cationic or amphipathic peptide sequences that can traverse mammalian plasma membranes and penetrate the BBB. These are used as efficient vehicles to various therapeutic loaders into brain tissue in the CNS disease management.^{107,108} Its limitations are the lack of specific target, i.e., unwanted peripheral effects may result.

Cell-mediated delivery

Recent reckoning have uses of 2 types of cells, i.e. immunocytes and stem cells, as cellular Trojan horses to carry hidden therapeutic loaders across the BBB. Neural stem cells and mesenchymal stem cells are the 2 stem cells having brilliant property to attract toward malignant brain cells. These are harmless in various applications and it is very convenient to cultivate and transplant them. Genes, NPs, enzyme-prodrug assembly and many more are exploiting this cargo system for effective therapeutics.¹⁰⁹⁻¹¹¹

Strategies to bypass the BBB

Intranasal drug delivery

Intranasal drug or biomolecules delivery is an efficient plan to bypass the BBB.¹¹² It is non-invasive, safe, and simple. The effectiveness of intranasal delivery is determined by administration factors and physicochemical properties, such as the patient's head position, dosing device, drug volume, pH value, osmotic pressure, and drug solubility. It has been used earlier in the diphtheria treatment. Thus, it proves it as a reliable administration route.¹¹³ Penetration enhancers, nanoparticles and adhesion agents nullifies the limitations associated with intranasal drug delivery which also increases the efficiency of drug delivery. Successful delivery of stem cells using the intranasal approach as a therapy for experimental allergic encephalomyelitis in rats, an animal model of multiple sclerosis have been achieved.¹¹⁴ Nasal glucagon like peptide-1 has already been used in patients.¹¹⁵ This is a promising development for patients with diabetes, and has the potential that insulin may be administered in a similar

Table 3. Various strategies to cross BBB

Strategy	Pros	Cons	Clinical trial
Nanotechnology	Sustained cargo release Easy parenchymal uptake Excellent penetrating ability	Fast removal	Phase II
CPPs		Fast removal Non-specificity Immunoresponsive ↑ intracranial pressure Necrosis Tissue damage	Phase II
Hyperthermia	Low cellular toxicity Easy to perform Drug compatible		Phase I
Cell mediated delivery	Low cytotoxicity Controlled drug release Targeted transport	Require high cell quantity Cell injury	Phase I
RMT	Site-specific	Low dissociation rate Rapid cargo degradation Possible toxicity	Phase III

way. Future research is needed to further reveal the mechanisms of nasal drug delivery and at the same time improve the technology and solution preparation. This will achieve a better targeting, improved effectiveness, and higher drug concentrations.

Intracarotid infusion

Intracarotid infusion of hypertonic solutions, facilitated by Ca^{2+} mediated contraction of the actin cytoskeleton allows transient opening of BBB. The hypertonic solution consist of mannitol, arabinose, etc. The vasodilation and shrinkage of cerebral capillary endothelial cells results in BBB opening. Therefore the opening of the paracellular route allows free drug diffusion across BBB.¹¹⁶

Pathogens and related components are able to reach the CNS by direct transcellular passage across the BBB through paracellular path by carriage within peripherally circulating white blood cells which are involved in immune surveillance of the CNS i.e., Trojan horse, through the retrograde neuronal axons, or through regions of the brain with distinct BBB physiology. Rabies virus glycoprotein (RVG), Chlorotoxin (CTX), Dendrotoxin, Conantokin-G, Tetanus toxin, and Hannah toxin are some examples of CNS-directed delivery achieved by pathogenic strategies.²²

Transmucosal drug delivery

Nowadays methods to bypass the BBB, such as pharmacologic modification and direct transcranial catheter implantation, are very costly, with lot of complications, and infeasible to fulfill chronic needs of a large, aging patient population. Transmucosal drug delivery is an innovative method of direct CNS drug delivery using heterotopic mucosal grafts. This method is based on established endoscopic skull base nasoseptal flap reconstruction techniques. The model has successfully demonstrated CNS delivery of chromophore-tagged molecules 1000 times larger than those typically permitted by the BBB.¹¹⁷

Development of *in vitro* BBB model systems (brain-on-a-chip)

Organs-on-chips are microfluidic devices for culturing living cells in continuously perfused, micrometer-sized chambers in order to model physiological functions of tissues and organs. The aim is to synthesize minimal functional units that recapitulate tissue and organ-level functions. This helps to understand the basic mechanisms of organ physiology and pathophysiology. It's also useful to study the biological processes that depend on tissue micro-architecture and perfusion, and that consumes less than one month duration pathophysiological processes. Researchers have fabricated chips for the study of the blood-brain barrier.¹¹⁸⁻¹²² All the *in vivo* situation characteristics are displayed by these fabricated chips. It is costly and some other settlements have to be accepted are predictive value, time and capacity. The criteria to meet for an ideal BBB model are: it should be reproducible for solute permeability, it should display restrictive paracellular pathway and real physiological architecture, it should give expression for functions of transporters, and there should be ease of culture.¹²³ Although of several advantages of different *in vitro* BBB models, there exists some limitations too which are enlisted in Table 4.

To mimic brain diseases

Neuro-inflammation was studied on chips by stimulating the vascular endothelium with $\text{TNF-}\alpha$, which activated adjacent microglia and astrocytes, similar to what occurs *in vivo* in situations such as neuro-infectious disease. In a study synapse was modeled and was studied for their formation in neurons and diseased cells. The presence of diseased cells had a great effect on the number and stability of synaptic contacts.¹²⁴ Synapse formation at the neuromuscular junction was also examined in chips containing mouse embryonic stem cell-derived motor neurons and C2C12 myotubes.¹²⁵ Myelination has been studied on chips by co-culturing Schwann cells derived from human embryonic stem cells

with human axons.¹²⁶ Co-cultures of different neural cells have been used to model neurological diseases on chips. In amyotrophic lateral sclerosis genetically modified neurons and astrocytes overexpressed either the wild-type or a mutated form of human super oxide dismutase enzyme 1 (SOD1), were cultured in close proximity but with no cell-cell contact.¹²⁷ Experiments were carried out to understand the role of West Nile virus (WNV) infected leukocytes versus endothelial cells in BBB disruption using the transmigration of either WNV-infected monocytes at day 2 after infection across the uninfected BBB models or uninfected monocytes across the WNV-infected BBB models at day 3 after infection.¹²⁸ Combination of stem cell delivery, heat-inducible gene expression and mild heating with high-intensity focused ultrasound (HIFU) under MRI guidance are employed to remotely permeabilize BBB by using brain on chip intro model.¹²⁹ Dynamic *in vitro* blood-brain barrier (DIV-BBB) deals with endothelial cells cocultured with albuminal astrocytes under flow condition which develop a phenotype similar to that of the brain microvascular EC in situ. DIV-BBB incorporates modified hollow fibers have transmural microholes (2 to 4 μm $\varnothing$) allowing for the trans endothelial trafficking of immune cells. This model has shown promising results in dealing BBB in neuroinflammatory diseases.¹³⁰

Researchers have described procedures that can be utilized for both freshly isolated mouse brain microvascular ECs (MBMECs) and murine or human brain EC lines (bEnd5 or hCMEC/D3), cultivated either as a single monolayer or in cocultivation with primary mouse astrocytes (ACs). It comprises electrical assessment of the *in vitro* BBB exploiting a very recent device named cellZscope[®]. It also provides TEER (paracellular resistance) and cell membrane capacitance (Ccl-transcellular resistance), 2 independent measures of monolayer integrity. As various diseases are associated with BBB breakdown, BBB *in vitro* assays that generate reproducible results not only with primary brain ECs but also with EC lines.¹³¹ Researchers considered the *in vitro* BBB model as a boon by observing the performance of monocultured, cocultured, and triple-cultured primary cells and immortalized cell lines, including key parameters such as transendothelial

electrical resistance values, permeabilities of paracellular flux markers, and expression of BBB-specific marker proteins. Microfluidic systems are gaining ground as a new automated technical platform for cell culture and systematic analysis. It is very beneficial in dealing age-related CNS diseases.¹³²

To study transport mechanisms

A human blood-brain-barrier-on-a-chip was developed by lining a porous, fibronectin-coated polycarbonate membrane with human brain microvascular endothelium on one side and human astrocytes on the other.¹¹⁹ The device included embedded microelectrodes to measure trans-epithelial electrical resistance (TEER) across the barrier. TEER levels were 10 times higher in this chip than those of the identical cells grown on opposite sides of a polycarbonate membrane in a static transwell culture system. Co-cultures on the chip didn't allowed large molecules to cross than monocultures of endothelial cells. The barrier function achieved was approximately 25% of *in vivo* brain microvessels. This chip-model is better than conventional culture models for measuring the permeability barrier of the CNS. It also helps to understand the mechanism of drug passage across BBB and in CNS too. A multi-co-cultured microfluidic model of the neurovascular unit was also created by placing endothelial cells on one side of a porous membrane and a mixture of astrocytes, neurons and microglia on the other.¹²² During 10 day of culture the endothelium acquired good BBB function, and the neural cells generated both inhibitory and excitatory potentials.

Recently it is developed and characterized a highly reproducible rat syngeneic *in vitro* model of the BBB using co-cultures of primary rat brain endothelial cells (RBEC) and astrocytes to study receptors involved in transcytosis across the endothelial cell monolayer for the transport of rhodamine.¹²² Computer based *in silico* models could be the best candidates to improve cost and time efficacy. *In silico* models are potentially best suited for the development of drugs predicting efficacy and bioavailability in the CNS. Many pharmaceutical companies have already implemented this technology in their early drug development programs helping to identify well tolerated, safe and effective compounds.^{133,134}

Table 4. Advantages and disadvantages of different *in vitro* BBB models

Model	Advantages	Disadvantages
Epithelial cells over-expressing transporters	Cheap Easy to standardize	Differences between epithelial and endothelial cells Non-physiologically high levelsof transporters
Transwell monoculture	Uses brain endothelial cells Inexpensive	Effect of other cellular components of the NVU is neglected No shear stress
Co-culture	Takes into account the influence of other elements of the NVU	Relatively expensive and time-consuming No shear stress
Dynamic	Mimics <i>in vivo</i> situation Possibility of co-culture	Expensive no possibility to optically monitor the cells Special skills required to culture cells in these conditions
Microfluidic	Mimics <i>in vivo</i> situation Possibility of co-culture	Not well established models Presently expensive
In-silico models	Time and cost effective Highly reproducible Highly quantitative	Currently limited parameters limited complexities

In vivo (Multimodal) Imaging of Drug Delivery Devices for Real-Time Tracking

BBB permeability assessment using fluorescence imaging

This direct vessel imaging approach was developed in-house and is considered as a primary approach for detecting BBB disruption events *in vivo*.¹³⁵ Semi-quantifying BBB permeability in anesthetized animals is its major expedience. Its validation was achieved in 2010 when its application enabled the detection and quantification of major BBB dysfunction following the use of deoxycholic acid¹³⁶ (DOC) or arterial stroke applied with photothrombosis.¹³⁷

Laser-speckle imaging for BBB permeability assessment

In recent time, laser speckle contrast imaging (LSCI) has been widely used to measure blood flow in a variety of tissues. When coherent laser illuminates an object the beam is distributed to split, and adds constructively as well as destructively. A random interference pattern can be visualized with appropriate optical receptors.¹³⁸ When the moving objects like blood cells are illuminated by laser the pattern fluctuates with time. Reduced spatial contrast in areas of increased motion was found from the integrated results. Therefore any live or moving particles can be visualized via LSCI. Contrast enhanced nuclear imaging (CENI) can quantitatively assess cellular processes. Additionally, PET with the administration of 18F-2-fluoro-2-deoxy-d-glucose (18F-FDG) is widely used to quantify changes in glucose metabolism.^{139,140}

Tomographic Methods

Recently, positron emission tomography (PET) and single-photon emission-computed tomography (SPECT) have been employed to study brain uptake kinetics, cerebral blood flow, BBB integrity, and efflux mechanisms.¹⁴¹ It involves a small amount of labeled compound with a positron-emitting radionuclide injected intravenously and allowed to distribute to the different tissues in the body. The emitted γ radiation is then measured as a function of tissue depth by the instrument. Computer software is employed to create a 3-dimensional image of the distribution of the substance in the brain and other tissues. PET gives higher-resolution images than the SPECT. Both are non-invasive techniques and can be used on both human and animal subjects. The possibility to obtain excellent spatial resolution is its main advantage.¹⁴² But some of disadvantages are also associated with it i.e. costly instrumentation and use of synthesized radiolabelled agents. PET utilizes ¹¹Cor¹⁸F-labeled compounds, while SPECT exploits ¹²³I. The 20 min short half-life of ¹¹C-labeled compounds suggests its synthesis on the spot prior to administration. Radioactive fluorine or iodine labeled compounds has longer half-lives, but they can mislead the results due to different transport properties. It is not possible to determine the free fraction in comparison to bound fraction *in vivo*. Undistinguished transport of the drug and its metabolites is also one of its limitations because it measures only radioactivity.⁶⁴

Nearly all CNS drugs must travel the brain extracellular space (ECS) to exert their effects. Diffusion controls distribution

within the ECS and is inspired by properties of the brain micro-environment as well as the specific characteristics of the diffusing molecule. To measure extracellular diffusion *in vivo* the techniques widely used are real-time iontophoresis, ventriculocisternal perfusion of radiotracers, and integrative optical imaging (IOI) of fluorescent probes.¹⁴³ Different diffusion measurements have revealed that all molecules experience some occlusion as they travel through the brain ECS and experience cellular obstacles. This hindrance is calculated as tortuosity ($\lambda = (D/D^*)^{1/2}$, where D is the free diffusion coefficient and D^* is the effective diffusion coefficient in brain.^{143,144} It is a dimensionless parameter. Real-time integrative optical imaging has been used to measure the diffusion properties of fluorescently labeled, non-targeted IgG after pressure injection in both free solution and in adult rat neocortex *in vivo*, revealing IgG diffusion in free medium is approximately 10 times greater than in brain ECS.^{145,146}

A novel bimodal iron oxide particle is validated as an alternate of ferumoxides for efficient labeling of human neural stem cells (NSCs). The dextran-coated FeraTrack Direct (FTD)-Vio particles have extra far-red fluorophores for microscopic cell analysis. It utilizes MR relaxometry, spectrophotometric iron determination and microscopy for characterizing *in vitro* and *in vivo* revealing that FTD-Vio594 particles are safe and sensitive alternate of ferumoxides for longitudinal tracking of NSCs.¹⁴⁷

Recently Yin and co-workers assessed the effect of human adipose-derived stromal/stem cells (hADSCs) for treatment of ischemic stroke, and to demonstrate the feasibility of tracking transplanted HPF-labeled hADSCs in middle cerebral artery occlusion (MCAO)-injured rats by MRI *in vivo* targeting advancement of this technology for clinical purposes.¹⁴⁸ Recently researchers have used real-time integrative optical imaging to measure the diffusion properties of fluorescently labeled, non-targeted IgG after pressure injection in both free solution and in adult rat neocortex *in vivo*, resulting in IgG diffusion in free medium is ~10-fold greater than in brain ECS.¹³⁹

Conclusion and Future Prospects

As the nature have given us such legerdemain in the infrastructure of our whole body, especially the brain, the blood-brain-barrier was always been a typical challenge for the health researchers because of its very concise, tight, complex and web like anatomy. It is true that since last few decades the different advanced imaging techniques have been developed, so as it is more convenient to understand the most aspects of materials and processes involved regarding BBB in healthy and diseased conditions via live tracking of biomolecules or drug in motion within it. By advanced development of brain-on-a-chip technique it is more convenient to save lot of time, capital and animal lives, which assures and makes this technique not only beneficial economically but also ethically. All these techniques being employed have the main goal to learn more and more deep about BBB development and pathophysiology concerned, so as to manage and eradicate the existing neurodegenerative and other brain diseases like Alzheimer and Parkinsonism which are still being a challenging

problem for the neurologists and related researchers. It is very requisite to develop strategies for efficient cargo delivery of drugs or biomolecules or genes with their relevant carriers which could bypass or even cross the BBB for intended therapeutic response in diseased condition. *Charak Samhita* and *Sushruta Samhita* (Ancient text or compendium of Ayurveda or Indian Traditional medicines) have evidence of therapeutic importance of sniffing powdered natural herbs and *ghee* (a clarified form of butter) through nasal route. Also the natural drugs like withanolides, brahmins and curcumin have shown very good results in treating various brain related disorders. Therefore we would like to suggest for more optimization of intranasal drug delivery system without avoiding the importance of natural drugs along with the developing modern nanocarriers and instrumentation techniques. The use of alternative route is suggested, as it would ameliorate delivery of drugs directly to brain as well as would not allow the exposure of drugs to the unwanted tissues of the body. The

traditional routes of delivery such as oral route has its own challenges such as first pass metabolism and exposure of the drugs to the varying pH conditions of the GIT. The use of nanotechnological carriers would further synergize the effectivity of delivery of drugs to brain through the nasal route.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Funding

The authors are grateful and would like to acknowledge the University Grants Commission (UGC) New Delhi, India and Science and Engineering Research Board (SERB), Department of Science and Technology (DST), New Delhi India, for providing research funding.

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