

Review

# Overcoming the Blood–Brain Barrier. Challenges and Tricks for CNS Drug Delivery

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**Abstract:** Treatment of certain central nervous system disorders, including different types of cerebral malignancies, is limited by traditional oral or systemic administrations of therapeutic drugs due to possible serious side effects and/or lack of the brain penetration and, therefore, the efficacy of the drugs is diminished. During the last decade, several new technologies were developed to overcome barrier properties of cerebral capillaries. This review gives a short overview of the structural elements and anatomical features of the blood–brain barrier. The various in vitro (static and dynamic), in vivo (microdialysis), and in situ (brain perfusion) blood–brain barrier models are also presented. The drug formulations and administration options to deliver molecules effectively to the central nervous system (CNS) are presented. Nanocarriers, nanoparticles (lipid, polymeric, magnetic, gold, and carbon based nanoparticles, dendrimers, etc.), viral and peptid vectors and shuttles, sonoporation and microbubbles are briefly shown. The modulation of receptors and efflux transporters in the cell membrane can also be an effective approach to enhance brain exposure to therapeutic compounds. Intranasal administration is a noninvasive delivery route to bypass the blood–brain barrier, while direct brain administration is an invasive mode to target the brain region with therapeutic drug concentrations locally. Nowadays, both technological and mechanistic tools are available to assist in overcoming the blood–brain barrier. With these techniques more effective and even safer drugs can be developed for the treatment of devastating brain disorders.

**Keywords:** structure of the blood–brain barrier; models of the blood–brain barrier; drug delivery across the blood–brain barrier; nanocarriers; nanoparticles; vectors; intranasal delivery; efflux transporter inhibition; ultrasound-microbubbles

## 1. Introduction

The capillary microvessels of the brain have evolved to constrain the movement of molecules and cells between blood and brain, providing a natural defense against circulating toxic or infectious agents. The relative impermeability of the blood–brain barrier (BBB) results from tight junctions and adherens junctions between capillary endothelial cells formed by cell adhesion molecules. Brain endothelial cells also possess few alternate transport pathways (e.g., fenestra, transendothelial channels, pinocytotic vesicles), and express high levels of active efflux transport proteins, including P-glycoprotein (P-gp, MDR-1 or ABCB1) and breast cancer resistance protein (BCRP, ABCG2). The BBB maintains essential brain homeostasis but as a result, represents a significant impediment to the effective treatment of many brain diseases [1,2].

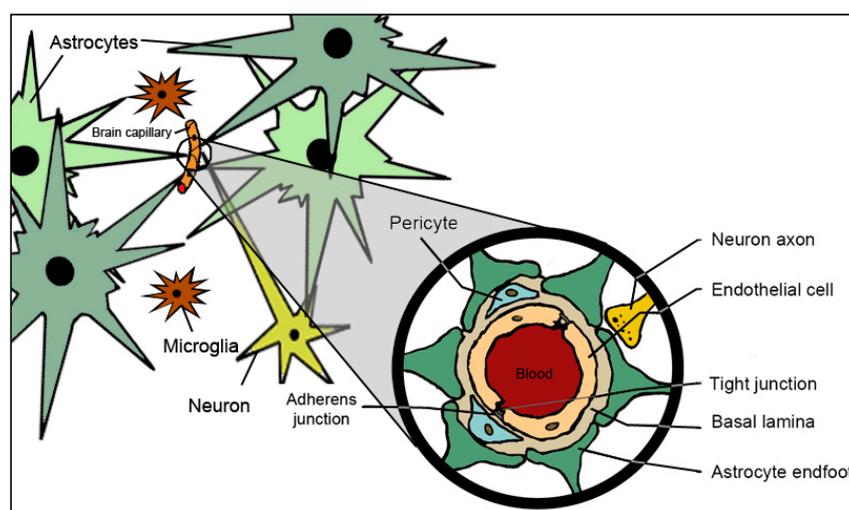
Current strategies to enhance drug delivery to the brain are either focused on locally circumventing the BBB through direct injections or nasal drug applications, for example, or globally through the bloodstream (using targeted delivery approaches or by opening the blood–brain barrier). Many approaches to enhance drug delivery across the BBB are under development, both by academic

research groups as well as pharmaceutical and biotechnology companies. To translate basic (academic) research into safe and effective treatments for patients with devastating brain diseases, many steps in many different research areas are required. The pharmaceutical formulation (chemistry, manufacturing, control, analytics, and selection of proper nanocarriers), pharmacology and pharmacokinetics (drug delivery, neuroscience, and crossing the blood–brain barrier), and safety (toxicology, behavioral, route of drug administration, and chronic treatment) concerns should be harmonized and taken into account. One of the major challenges in the area of brain delivery is first to find an efficient vector for brain delivery using a physiological pathway mechanism to cross the BBB. These vectors can be in the form of peptides, proteins, antibodies, or some other specific formulation, such as nanoparticles, which target a specific receptor at the BBB and will cross the BBB by transcytosis.

In this review, the authors give an overview of the different advanced technologies and tricks on how to cross the BBB, and how to deliver pharmacologically active molecules to the central nervous system (CNS) target site. This article attempts to provide a synthesis of the existing knowledge of the observations and findings from the last decades on the structure and function of the BBB and classical technological approaches to overcome this physiological barrier. It also includes the newest developments and methods for modeling and crossing the blood–brain interfaces (e.g., dynamic chip models of the BBB, nanocarriers, vectors, and efflux transporter modulation to help the brain uptake of therapeutic drugs).

## 2. Anatomy, Physiology and Molecular Constituents of Blood–Brain Barrier

The mature blood–brain barrier (BBB) is composed of capillary endothelial cells (ECs) tightly connected with tight junctions (TJs) and adherens junctions (AJs) that prevent paracellular transport [3] and have a low pinocytotic activity [4,5], although limited transcellular transport does occur [6]. In addition, the BBB is influenced by closely associated perivascular astrocytic end-feet, pericytes, and microglia [7] (Figure 1). These different cell types play essential roles in BBB induction and maintenance by regulating the proliferation, migration, and vascular branching of the brain endothelial cells. Additionally, the basement membrane provides structural support around the pericytes and endothelial cells. The basal lamina is contiguous with the plasma membranes of the astrocyte end-feet [8]. The BBB provides strong resistance to movement of ions, with transendothelial electrical resistance (TEER) around  $1500 \times \text{cm}^2$  [5], 100 times higher than that for peripheral microvessels [9,10].

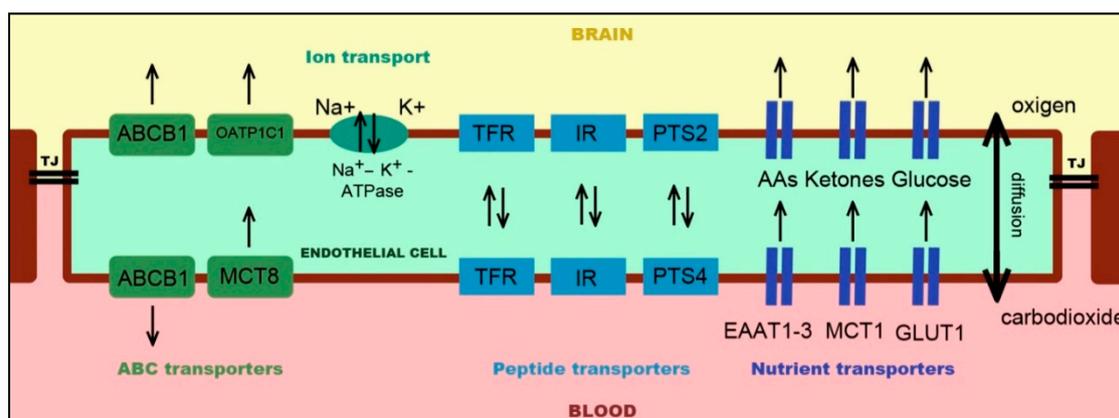


**Figure 1.** Schematic structure of the blood–brain barrier (BBB). The brain capillary endothelial cells (ECs) connected to each other by tight junctions (TJs) and adherens junctions (AJs). The endothelial cells are surrounded by basal membrane which also covers the connecting pericytes. Around the brain microvessels astrocyte endfeet are also essential providers of the barrier function. Additional supporting cell types are microglia cells and the connecting neurons.

### 2.1. Endothelial Cells

Compared to peripheral vasculature, BBB ECs are characterized by increased mitochondrial content, exhibit minimal pinocytotic activity, and lack fenestrations [11–14]. The restricted paracellular permeability of the capillary EC layer is warranted by two intercellular molecular binding systems: the AJs and the TJs. TJs are dynamic complexes of multiple protein constituents including junctional adhesion molecules (JAMs), occludin, claudins (i.e., claudin-1, -3, and -5), and membrane-associated guanylate kinase (MAGUK)-like proteins (i.e., ZO-1, -2 and -3) [8]. AJs are composed of multiple protein components including vascular endothelium (VE) cadherin, actin, nectin, and catenin [15]. Both on the basolateral and on apical surfaces of ECs there are different types of transporter proteins expressed.

The schematic overview of the most important transporter systems located in the BBB ECs is presented in Figure 2.



**Figure 2.** The schematic representation of the most important transport systems at the blood–brain barrier endothelial cells.

### 2.2. Astrocytes

Astrocytes are glial cells that help, support and protect neurons by controlling neurotransmitter and ion concentrations to maintain the homeostatic balance of the neural microenvironment, by modulating synaptic transmission and by regulating immune reactions [16] and by interacting with endothelial cells through their endfeet projections that encircle the basolateral side of cerebral capillaries [17].

### 2.3. Pericytes

Pericytes regulate (1) BBB integrity, i.e., tight or adherens junctions and transcytosis across the BBB; (2) angiogenesis, i.e., microvascular remodeling, stability, and architecture; (3) phagocytosis, i.e., clearance of toxic metabolites from the central nervous system; (4) cerebral blood flow, capillary diameter; (5) neuroinflammation, i.e., leukocyte trafficking into the brain; and (6) multipotent stem cell activity [18].

### 2.4. Microglia

Microglia, the primary immune cells of the brain, are ubiquitously distributed in the CNS and are activated in response to systemic inflammation, trauma, and several CNS pathophysiologicals [12,19–21]. Microglial activation in response to pathophysiological stressors can trigger changes in cell morphology, which include reduced complexity of cellular processes and transition from a ramified morphology to an amoeboid appearance [19].

Activation of microglia is associated with altered TJ protein expression and increased BBB permeability [22]. Microglia are a supportive cell type for the proper function of the BBB.

### 3. In Vitro, In Situ, and In Vivo Models for Testing the Blood–Brain Barrier

#### 3.1. In Vitro Modeling

##### 3.1.1. Static In Vitro BBB Models

The simplest in vitro BBB model consists of a monoculture of cerebral endothelial cells seeded on a semi-permeable support under static culture conditions.

*Brain endothelial cells.* The brain endothelial cells used in in vitro BBB modeling can be primary cells or immortalized cell lines. The most frequently used primary brain endothelial cells are isolated from rat, mouse, bovine, porcine, and human [23]. Primary cells might not be a convenient choice for every in vitro study/testing due to limited availability, high costs, time-consuming preparations (including the necessity for special skills required for the cellular isolation), and cultures being susceptible to internal and external contamination [23,24]. On the other hand, immortalized cell lines remain viable over many passages with a higher experimental reproducibility between tests compared with primary cells [25]. Thus, making these cells relatively reliable, easily accessible, and affordable considering that culture preparation time and costs are reduced [26]. The most frequently used cell lines for modeling the BBB are summarized in Table 1.

**Table 1.** Immortalized cell line models of BBB (modified from Abbot et al., 2014 [23]).

Cell Line	Species, Transfection	Recent References
RBE4	Rat (2)	Branca et al., 2018 [27]
hCMEC/D3	Human (5)	Kuroda et al., 2018 [28]
MBEC4	Mouse (1)	Mizutani et al., 2016 [29]
*bEND3	Mouse (3)	Zhou et al., 2018 [30]
TR-iBRB2	Rat retina (4)	Kinoshita et al., 2018 [31]
GP8.3	Rat (1)	Motta et al., 2015 [32]
GPNT	Rat (1)	Suzuki et al., 2016 [33]
TR-BBB13	Rat (4)	Tega et al., 2018 [34]
RBEC1	Rat (1)	Ishisaka et al., 2014 [35]
cEND	Mouse (3)	Blecharz–Lang et al., 2018 [36]
*bEND5	Mouse (3)	Zuccolo et al., 2017 [37]
SV-HCEC	Human (1)	Dasgupta et al., 2011 [38]

Transfection vector/method (1) SV40 large T antigen; (2) Adenovirus E1A gene; (3) Polyoma virus middle T antigen; (4) Transgenic (Tg) rat or mouse harboring temperature sensitive SV40 large T antigen; (5) Sequential lentiviral transduction of hTERT and SV40 large T antigen. \* Available from ECACC (European Collection of Animal Cell Cultures) and ATCC (American Type Culture Collection)

From in vitro co-culture models, generally, co-cultures of cerebral endothelial cells with astrocytes and pericytes (bi- or triple co-cultures) are widely used since they play a crucial role in the development of the paracellular tight junctions of the BBB and modulating endothelial cell functions [24,26].

*Astrocytes.* Although they are not in direct contact with endothelial cells in the neurovascular unit due to the presence of the basal membrane astrocytes, the interaction between these two cells is the best characterized (for review see refs Abbot et al., 2006 and Alvarez et al., 2013 [17,39]). The effect of astrocytes during adulthood on the inter-endothelial junctions is especially important as it largely determines permeability and also on the modulation of transporter expression. Astrocytes play a crucial role in the development of the complexity of tight junctions [40] and upregulation of the efflux transporters ABCB1 [41] and ABCG2 [24,42].

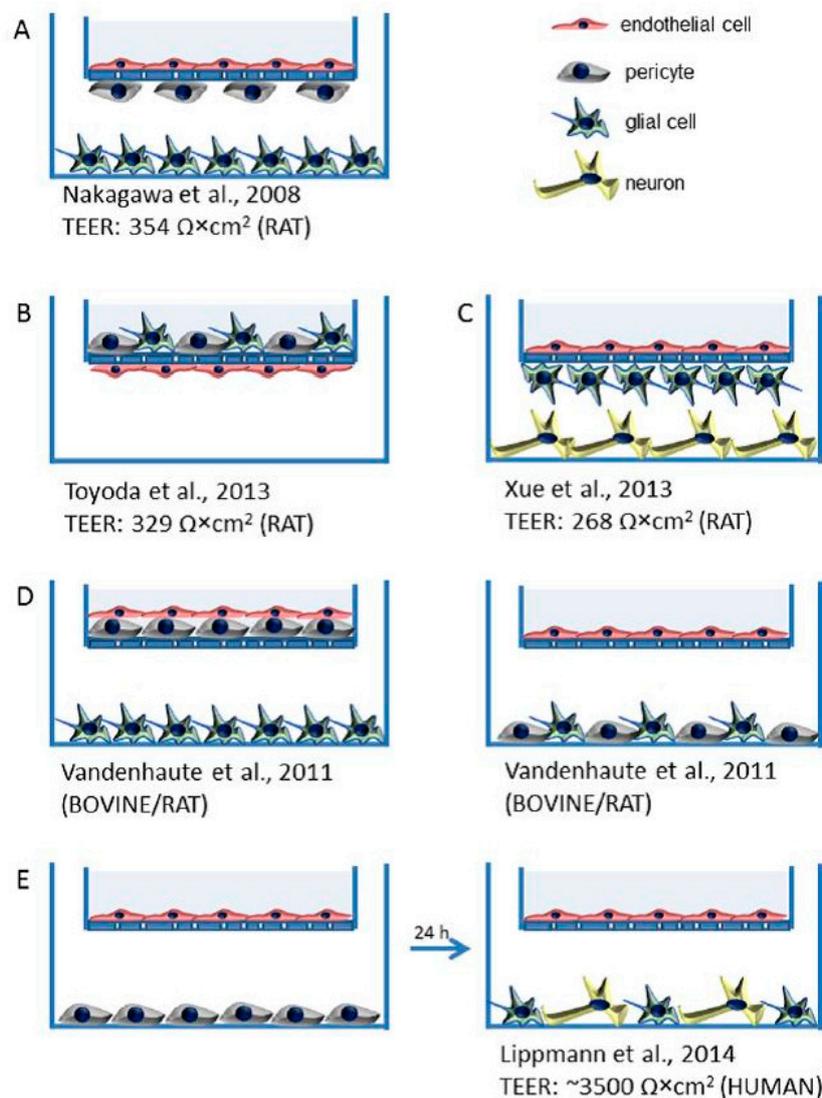
*Pericytes.* Although pericytes are the closest neighbors of endothelial cells in vivo, their effect is far less well characterized than that of the astrocytes. They have multiple roles, such as contractile,

immune, phagocytic, and angiogenic functions. Pericytes also contribute to the formation of the basement membrane by synthesizing laminin, type IV collagen, and glycosaminoglycans. Besides being important elements of the BBB, they are a source of adult pluripotent stem cells as well [24,43].

*Stem cells.* Stem cells are a promising source of cells for the generation of in vitro human BBB models because these cells have the capacity to differentiate into cerebral endothelial cells, they can give rise to a significant number of BBB cells.

Embryonic stem cells (ESCs) are an alternative pluripotent stem cell therapy option due to their ability to differentiate into various types of brain cells in addition to their indefinite self-renewal abilities in vitro [44]. Neural stem cells (NSCs) are multipotent stem cells in adult brains that, unlike ESCs, have a decreased potential of self-renewal and normally, for the purpose of repair, differentiate into only one cell lineage of the tissue [45]. NSCs can differentiate into neuronal cells and, hence, have huge potential for the generation of in vitro human BBB models featuring a more complex neurovascular unit (NVU) system encompassing both vascular and brain tissue [26,45]. Induced pluripotent stem cells (iPSCs) are potential stem cells that can be used as a replacement therapy for human cellular models. The two main advantages of iPSCs are the avoidance of the use of ESCs for ethical reasons and the ability to be generated from the patients themselves [46]. The use of mesenchymal stem cells (MSCs) offers huge potential for application in the treatment of brain diseases, not only because MSCs are easily isolated, but they can also be easily expanded from tissues without ethical concerns. Recent work has shown strong similarities between MSCs and pericytes [47]. Similar multipotential stem cell activity has been exhibited in CNS microvascular cells to that seen in MSCs and also pericytes and MSCs express many of the same cell surface markers [48].

To enable drug transportation studies, advances in the culture setup have been made, resulting in cell culture on a filter membrane suspended in a well, the so-called Transwell system [23]. The Transwell system is essentially a side by side vertical diffusion system which comprises a microporous semipermeable membrane (on which cells can be seeded submerged in feeding medium [49] that separates the vascular and parenchymal side compartments. Transwell systems are ideal for linear kinetic studies of transport due to the fixed volumes of each compartment, but there are substantial limitations inherent to these platforms that need to be taken into consideration. For example, the lack of a three-dimensional structure present in vivo and lack of endothelial exposure to physiological shear stress which limits the differentiation of the endothelium into a BBB phenotype (or maintenance of BBB properties in fully differentiated cells) [26]. The possible seeding arrangements can be seen in Figure 3.

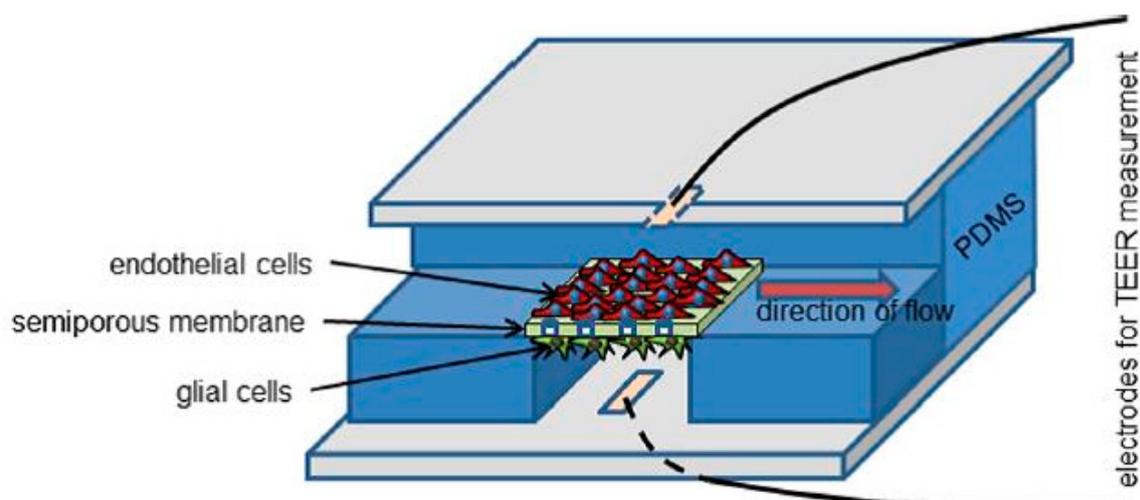


**Figure 3.** In vitro BBB models using three different cell types. Cerebral endothelial cells are cultured on semi-porous membranes in the presence of pericytes, astrocytes, and/or neurons in different arrangements. (From Wilhelm and Krizbai, 2014 [24] with permission). Panel A [50], B [51], C [52], D [53], E [54].

### 3.1.2. Dynamic In Vitro BBB Models

In dynamic systems, brain endothelial cells are cultured in the lumen of hollow fibers inside a sealed chamber and are exposed to flow, while the NVU is seeded in the extraluminal compartment. Intraluminal flow is generated by a variable-speed pulsatile pump that can be regulated to produce desirable intraluminal pressure physiologically comparable to that observed in capillaries in vivo. Low permeability to intraluminal polar molecules, high TEER, negligible extravasation of proteins, expression of specialized transporters, efflux systems, and ion channels are several significant advantages of dynamic in vitro BBB models.

Microfluidic-on-chips as a type of dynamic 3D model and novel class of micro-engineered laboratory models of BBB combine several advantages of current in vivo and in vitro models [55,56] (see also Figure 4).



**Figure 4.** Schematic representation of a microfluidic model. Cerebral endothelial and glial cells are cultured on the two sides of a semi-porous membrane placed at the interface of two microchannels. (From Wilhelm and Krizbai, 2014 [24] with permission).

In a typical tissue-on-a chip embodiment, microfluidic channels are fabricated using soft lithography techniques by molding an elastomeric material, polydimethylsiloxane (PDMS), against a photo-defined master mold and a porous cell culture substrate is then sandwiched and sealed between the channel networks [57]. This system improves BBB modeling by having more realistic dimensions and geometries [58–60].

### 3.2. *In vivo* Modeling

Because of the complexity of the BBB and the realization that many BBB properties are not constant but vary with disease, development, and drug exposure, it has been difficult to develop a small toolbox of *in vitro* or *in silico* models that adequately predict drug transport and availability to the CNS. As a consequence, *in vivo* testing of brain drug uptake and equilibration is still considered the “gold standard” of any CNS drug delivery program.

*In vivo* CNS drug penetration experiments are designed along classic pharmacokinetic lines to measure two primary parameters: the rate at which a compound crosses into brain (i.e., BBB permeability-surface area product (PS)) and the extent to which the compound distributes within the CNS (i.e., brain distribution volume or partition coefficient ( $K_p$ , brain)) [23,61,62].

It can be important to distinguish the concentration of drug that is free or unbound from the total drug concentration in brain and serum because the free drug concentration correlates best with pharmacodynamics models of activity. Many drugs bind significantly to proteins and lipids based upon lipophilicity and other factors. In such cases, total concentration can differ greatly from the free drug concentration that is usually the driving force for drug diffusion and equilibration.

The unbound fraction of drugs ( $f_u$ ) can be determined from total concentration by *ex vivo* equilibrium dialysis or ultrafiltration [61]. Once  $f_u$  is measured, the free concentration ( $C_u$ ) can be calculated as

$$C_u = f_u \times C_{tot} \quad (1)$$

$C_u$  can also be measured directly in the brain by *in vivo* cerebral microdialysis [61,63]. The possible drug–BBB transporter interactions can also be verified by microdialysis with the determination of  $AUC_{brain}/AUC_{blood}$  ratio in the transporter substrate or substrate plus inhibitor-treated animals [64,65].

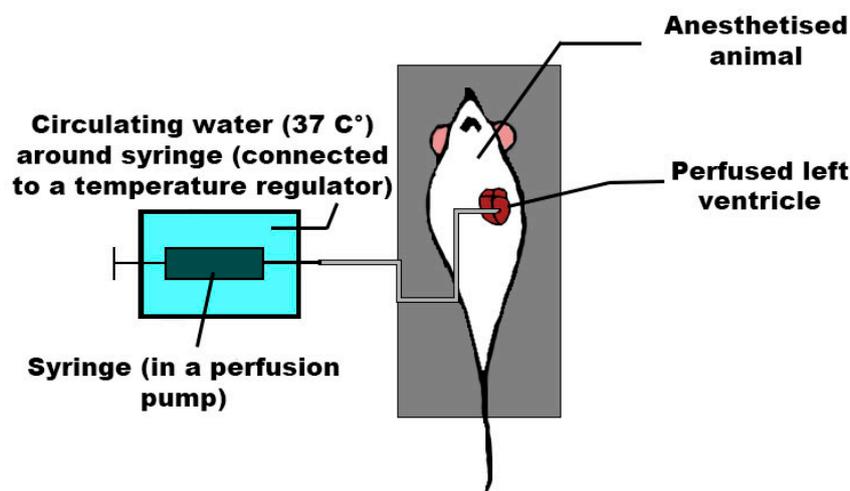
The permeability of BBB is changed in certain types of brain pathologies, such as malignant gliomas, ischemic or hemorrhagic stroke or epilepsy. To mimic the malignant pathologic conditions many preclinical models have been developed. The early versions of genetically engineered mouse

(GEM) models of brain tumors were based on the constitutional inactivation of tumor suppressor genes and/or the introduction of activated oncogenes into the germline. Later the contemporary GEM possessing produced inducible tumor suppressor gene knockouts, oncogene knockins, and improved cell type-specificity control over genetic alteration induction [66,67]. Although in recent years the neuro-oncology research community has directed more attention to the use of patient-derived xenograft models for therapeutic testing, the syngeneic, immunocompetent rodent models and the GEM models continue to serve a critically important role in brain tumor research, mainly for preclinical testing of therapies [66]. Many advanced techniques are used to assist and improve drug brain-tumor exposure, for review see Raucher et al. 2018 [68].

### 3.3. In Situ Modeling

In some situations, additional information is required over the in vivo collected data regarding the mechanisms involved that restrict drug uptake into the brain at the BBB. With the normal in vivo approach, limits are placed on the degree to which an investigator can control or change brain blood flow and free drug concentration or block transport or metabolic mechanisms. Knockout animals are available for several key BBB transporters, such as p-glycoprotein, breast cancer resistance protein, multidrug resistance protein-4, and organic acid-transporting polypeptide. However, with current transporter knockouts, the alteration is not just at the BBB but at all sites within the CNS that usually express the transporter. This can lead to complexity in evaluating the separate role of the BBB in overall brain distribution of the compound.

As alternatives to direct in vivo analysis, in situ perfusion and brain efflux index methods are available for more specific studies of BBB transport. These approaches complement the standardised IV administration method but allow greater flexibility in studying factors that may alter transport [69]. The in situ perfusion method utilizes the in vivo structure of the BBB and cerebral tissues and simply superimposes its own vascular perfusion fluid in replacement of the animal's circulating blood (Figure 5). The particular key advantage of this method is the facile control of perfusate solute concentration which can be altered over a much greater range than generally tolerated in vivo.



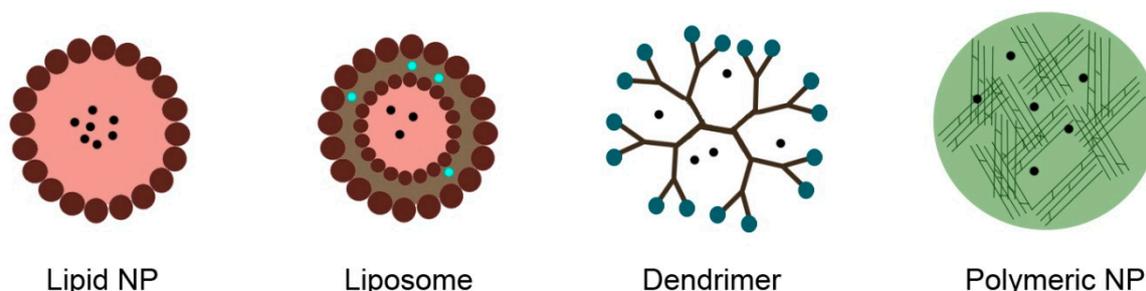
**Figure 5.** Typical setup for in situ brain perfusion with syringe, infusion pump, temperature control, and circulating water bath.

## 4. Focus on Drugs-Strategies to Improve Drug Delivery to the Brain

### 4.1. Nanocarriers, Nanoparticles, and Vectors

Some types of nanocarriers are presented in Figure 6. Size is a crucial parameter of the nanoparticles (NPs)—the delivery to the targeted tissue, the cellular uptake and the ability to reach

target proteins—as even chemical reactions are size dependent. The optimal size of a NP also depends on the specific location and type of targeted tissue [70], for example, most tumors have a vascular pore cutoff size between 380 and 780 nm [71]. However, Kang et al. have shown that for a gold NP has to be around 10 to 20 nm in diameter to effectively cross the BBB, while particles around 50 and 100 nm diameter were not distributed well enough into the brain [72,73].



**Figure 6.** Schematic representation of nanocarriers: a lipid nanoparticle, a liposome with lipophilic (cyan dot) and hydrophilic (black dot) drug, the dendrimer and a polymeric nanoparticle.

Size is also important to predict the elimination of the NP: Polymeric NPs larger than 200 nm are mostly sorted out by the spleen, NPs between 100 and 150 nm are captured by the Kupffer cells in the liver, and under 5.5 nm compounds leak out from the kidney [74]. Intravenously injected nanoparticles around 10 nm diameters have reached liver, spleen, kidney, testis, thymus, heart, lung and brain, while larger particles (50 nm, 100 nm, and 250 nm) were only primarily found in blood, liver, and spleen [75].

The shape of a carrier can influence its cellular uptake. For example, Kajino et al. tested the diffusion of spherical, cubic, rod-like, or worm-like shapes in the body with gold nanoparticles and Madden et al. with particle replication in non-wetting templates (PRINT) micro- and nanoparticles [76,77].

*Cyclodextrins (CDs)* are cyclic oligosaccharides made from starch [78]. There are three different variations of cyclodextrin: alpha- (consisting 6 glucopyranose), beta- (7GP) and gamma-cyclodextrin (8GP). The outer ring of CD is highly hydrophilic while the inside is more hydrophobic. Thus, CDs are perfect for delivering hydrophobic compounds into a hydrophilic environment, such as the blood. The different sizes and numbers of entrapping CDs can be optimized for trapping a wide variety of hydrophobic molecules [79].

In addition to their nanocarrier function, CDs also can be used as an active compound; CDs interact with lipid membrane rich in cholesterol and sphingolipids. It means CDs have cytotoxic and hemolytic properties due to their membrane alteration, such as increased fluidity and permeability [80].

To study the relationship between the blood–brain barrier and CDs Monnaert and coworkers used native, methylated, and hydroxypropylated CDs on a tight culture model [81]. Native CDs have significantly increased sucrose permeability. The lipid destructive effects were attenuated by methylation in the case of alpha-CD, while hydroxypropylation worked for only beta- and gamma-CDs. In addition, 12-alkyldimethylammonium-beta-CD (DMA-D(12)-CD) was shown to be non-toxic on cultured bovine brain endothelial cells at less than 10 nM concentration while having 30% of passage through BBB model [82]. Two methylated CDs RAMEB (randomly methylated-beta-CD) and CRYSMEB (partially methylated crystallized-beta CD) increased the transport of doxorubicin in bovine brain endothelia cells by extracting cholesterol from the cell membrane and, thus, altering the activity of a BBB efflux transporter, P-glycoprotein [83].

*Lipid nanoparticles (NPs).* Lipid NPs can be divided into two big groups: liposomes (LPs) and other lipid NPs, such as solid lipid NPs (SLN), and nanostructured lipid carriers (NLC) [84].

LPs are rather safe and selective therapeutic tools, composed of one or more lipid bilayers, and have been studied and used since the 1970s. They also can encapsulate lipophilic as well as lipophobic compounds in a wide variety of sizes [85,86]. However, non-targeted liposomes will

not effectively pass a healthy BBB, but with surface modification, for example, adding polymers, polysaccharides, peptides or antibodies, the characteristics and targets of LPs can be specified. Furthermore PEGylation is used to prevent liposomes from being eliminated by the immune system and improve biodistribution [87]. As for transporter-mediated crossing of the BBB, transferrin was added to the surface of LPs, to deliver gene therapy in Parkinson's disease animal models [88].

Cationic LPs are ideal for delivering drugs and genetic material due to their positively charged surface, which facilitates interaction with the cell membrane and enhances uptake. Their advantage is also their disadvantage: due to the cationic properties, peripheral tissues and serum proteins bind and block them from being able to pass the BBB [87]. Song and coworkers have constructed an ApoE-containing high density lipoprotein (HDL) inspired nanocarrier [89], which is BBB permeable [90] and has high A $\beta$ -binding affinity [91], for the treatment of Alzheimer's disease (AD).

SLNs are made of lipids which stays solid at body temperature. Their lack of the hydrophilic domain makes it possible to carry lipophilic compounds [84] but allows nanoparticle to cross the BBB with ease [92]. NLCs are modified SLNs that have improved drug loading capability and are more biocompatible.

A novel approach is on the horizon: *nanovesicles* derived from cells, so called exosomes, are potential new lipid nanocarriers of genes, proteins, and drugs [39,93,94]. Exosomes are a sub-population of specialized membranous extracellular vesicles derived from endocytotic compartments, are 30 to 100 nm in size, and are actively secreted by almost all cell types [95]. The exosomes ability to cross the BBB has been studied in zebrafish and mouse models [96,97]. Only exosomes isolated from brain endothelial cells could cross the BBB and deliver cargo into the CNS suggesting potential homo-tropism for the tissue of origin [97].

*Polymeric nanoparticles (PNPs)*. PNPs consist of matrix-like solid colloids, and can be found in a wide variety of sizes from 1 to 1000 nm. The polymers can have different natures: natural, such as albumin and chitosan or synthetic but every PNP is biocompatible and biodegradable [84].

Chitosan is a natural polysaccharide, derived from arthropods by the deacetylation of chitin and available in a wide variety of sizes/molecular weights and degree of acetylation and with surface PEGylation it also can cross the BBB and also can prolong its lifetime in the body [84]. Chitosan has minimal toxic properties and biocompatibility [98], but chronic administration is not advised [84].

Poly-butyl cyanoacrylate (PBCA), poly-lactic acid (PLA), and poly-lactic-co-glycolic acid (PLGA) are the most popular synthetics but are fully biocompatible and biodegradable polymers, especially PLGA, which has a more controllable drug release kinetics and better encapsulation properties [84]. PBCA can be produced easily but has weaker drug delivery capabilities. It has rapid biodegradation and insufficient absorption compared to more hydrophilic and lipophilic molecules [84]. PBCAs are usually targeted to low density lipoprotein (LDL) receptors to make them pass the BBB in mouse and rat models and deliver neural growth factor (NGF) [99] or Tacrine (also delivered by Chitosan carriers) [100] into the CNS as a drug for Alzheimer's and Parkinson's disease therapy.

Dendrimers (DDs) are macromolecules composed of three layers of polymers: 1) a central core; 2) branches, which are attached to the core and determine the generation of the DD (i.e., equals the number of layers the branches consist of. For example, a DD with three layers of branches is indicated as a G3 DD), and 3) the terminal functional groups, which create the surface of the nanoparticle [87]. The surface can be specialized for its function. For example, with transferrin conjugation, which enables the DD to cross the BBB [101], or with PEGylation or carbohydrate groups where toxic side effects and immune response can be excluded [102,103]. The most used DD is poly-amidoamine (PAMAM), which is water soluble and can encapsulate hydrophobe compounds. For example, cationic PAMAM DDs of different generations are used with PEGylation, as a safe in vivo drug delivery system in stroke patients [104].

There are some examples of successful use of DDs in CNS therapies: There was a study performed for a potential therapy to slow down and prevent Alzheimer's disease with cationic phosphorous DDs that disrupted amyloid  $\beta$  (A $\beta$ ) and MAP-Tau aggregations [105]. G0-PAMAM DD was used

with tetra-maleimidopropionyl conjugated and helical  $\beta$  peptide foldamers decorated on its surface and has been shown to successfully protect against  $A\beta$  induced long term potential, memory loss in Alzheimer’s disease [106]. G3 and G4 polypropylenimines with maltose coating, prevent  $A\beta$  fibrillation in mice [102].

Magnetic NPs (MNP) are nanoparticles with a metal core (mostly iron-oxide) that has an unpaired electron, therefore, has magnetic property. Iron is the most popular core material due to its low toxicity and easy elimination through the endogenous iron metabolic pathway and is usually used in oxide form because it is a more stable state [87]. Multiple coating is used to enhance drug delivery, such as polysaccharides (dextrin), polyethylene-glycol (PEG), phospholipids, peptides, for protection of the metal core from the body, improve pharmacokinetics, lower toxicity [107]. Fluorophores/radionuclides are used for basic research and for diagnostic purposes [108]. Iron-oxide MNPs can also be used as a contrast agents for magnetic resonance image (MRI), and therapy via magnetic fluid hyperthermia (MFH) [109].

Gold NPs (AuNP) are also a popular form of metallic core NPs. AuNPs are mostly coated covalently with an organic layer to improve biocompatibility, biophysical properties, and targeting. They are available in a wide variety of optical and electric features and structures, such as nanospheres and nanorods [110]. AuNPs have low toxicity and also easily cross the BBB [111]. For example, a wheat germ agglutinin horseradish peroxidase (WGA-HRP) conjugated AuNPs has been shown to be able to cross the BBB through intramuscular injection in the diaphragm of rats [112]. They are also used for imaging as X-ray contrast agents for computer tomography (CT) [113].

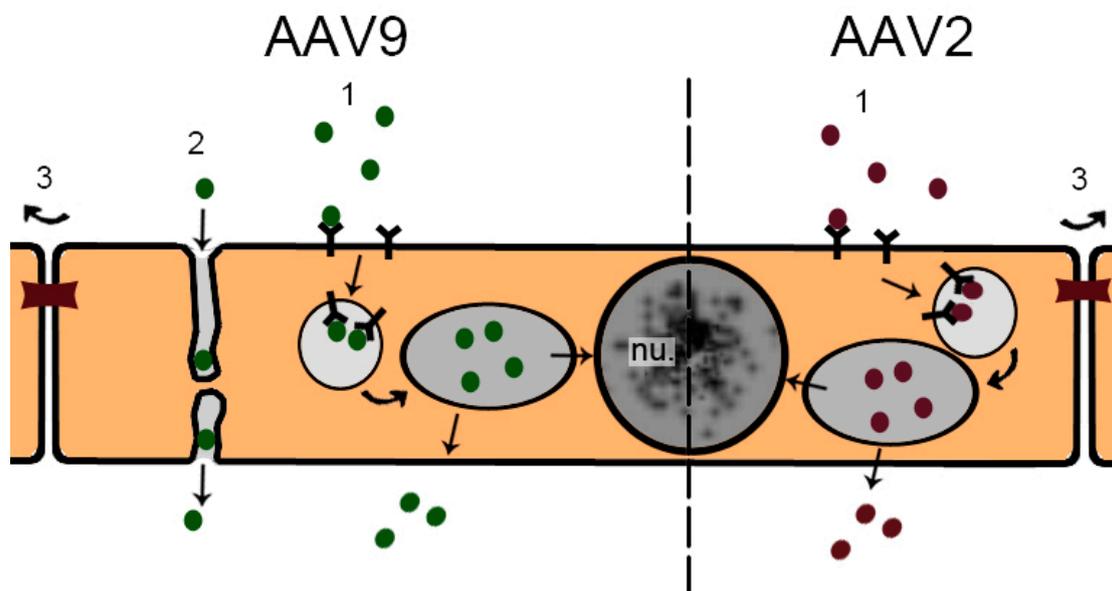
Carbon nanotubes (CNT) are made of graphene cylinders with open ends. The number of graphene layers can determine the flexibility of the carrier: fewer layers mean more flexibility [114]. CNTs are used as a chemotherapy drug, RNA, and protein delivery agent and also as biosensors [115]. As a CNS treatment possibility, a multi-walled carbon nanotube was functionalized with an amine group (MWCNTs-NH<sub>3</sub><sup>+</sup>) to effectively pass through the BBB via transcytosis in both in vitro and in vivo mouse models [116].

Some properties of nanocarriers and nanoparticles which are crucial for their penetration across the BBB are summarized in Table 2.

**Table 2.** Characterization of different nanocarriers/nanoparticles by size, charge and cerebral accumulation.

Name	Diameter (nm)	Accumulation	Charge	References
CD ( $\alpha/\beta/\gamma$ )	14.6/15.4/17.5	hydroxypropyl $\beta$ -CD: AUC 1.22, cmax 1.03	slightly positive	[117,118]
LP	<100; >200 decreased penetration into the brain	accumulate in ischemic brain	depends on lipid bilayer compounds	[119,120]
Cationic LP	65,5–352		positive	[121]
SLN	10–1000	38.4–42.7% in brain with Tf conjugation	Positive (stearylamine and glycol chitosan)	[120,122,123]
NLC nanovesicles	134–217 50–150		positive (HTCC)	[124,125]
Albumin (nanoparticle)	50–80	L-BSA and BSA NPs accumulate in intracranial tumor	negative	[128–130]
Chitosan	10–80	significant brain accumulation with RVG-Chito NP	positive	[131,132]
PBCA	145–250		negative	[133]
PLA	10–100			[120]
PLGA	90–150	unmodified PLGA NPs showed low brain uptake (<1%)	positive, near-natural and negative	[133–135]
Dendrimers (DD)	1–100		depends on surface groups	[120]
DD PAMAM	14–15	G6 PAMAM has high accumulation in brain of HCA dogs	positive	[136,137]
DD G13	24		depends on surface groups	[136]
MNP (Fe <sub>3</sub> O <sub>4</sub> )	10–300	up to 6 h no accumulation		[138]
AuNP	5–200	(with PEG) low		[139]
CNT	1 (diameter), >1000 (length)	MWNTs-NH <sub>3</sub> <sup>+</sup> penetrates the brain and accumulates	depending on crystalline order	[116,140]

Viral vectors are widely used for gene modification and delivery (Figure 7). This method can provide long-term expression of the transgene in non-dividing cells, especially with adeno-associated viral (AAV) vectors [141]. To get a sufficient quantity of therapeutic genes into the CNS, these vectors have to be engineered to be able to cross the BBB [142]. A major focus of recent gene therapy research has been in developing brain-tropic AAV vectors and understanding the mechanisms of their passage across the BBB. [143] AAV9 vectors are well tolerated IV viral vectors that can cross the endothelial cells of the BBB [144,145] via active-transport, while not compromising barrier integrity [146] and have promising results in clinical trials for their safety and their efficiency to transduce target cells, for example, in spinal muscular atrophy therapy [147–149], spinal muscle atrophy [150], and mucopolysaccharidosis IIIB (Sanfilippo syndrome) [151,152]. Zhang and coworkers showed that another four serotypes of recombinant AVV can also overcome the BBB: rAAVrh.10, rAAVrh.39, rAAVrh.43, and rAAV7 [153]. Simian virus 40 (SV40) is another safe to use viral vector for delivering genes into the CNS, mostly transducing cortical cells [154,155].



**Figure 7.** Adeno-associated viral 9 (AAV9) can cross the BBB by active-transport mechanism ((1) by receptor-mediated vesicular transport and (2) through tubular structures), while not compromising barrier integrity. Vesicular transport can end up in the nucleus (nu.) or on the basolateral side of the endothelial cells. Viral vectors that cannot pass through the BBB on its own (such as AAV2) can use the receptor-mediated route if the surface has been provided with a receptor for the ligand. Neither AAV9 nor AAV2 can pass between endothelial cells (3) because of the barrier of tight junctions (TJ).

If the vector itself cannot pass the BBB, there are two alternative solutions: 1) get the vectors through after the transient disruption of tight junctions in the endothelial microvasculature, 2) or by receptor-mediated transcytosis [142].

With the transient disruption of the BBB (tight junctions), viral vectors are able to enter by paracellular transport into the CNS. This disruption can be accomplished by osmotically shrinking the cells of the BBB, for example, with high concentration of mannitol [143]. Mannitol does not penetrate the cell membrane and is quickly eliminated from the body by the kidney. This method has also been used for increasing the transport of chemotherapeutic drugs to treat brain tumors [156,157]. Mannitol-mediated osmotic disruption has been combined with viral vectors, such as adenovirus (Ad), herpesvirus, adeno-associated virus (AAV, AAV2 naturally does not cross the barrier), and SV40 [60,64,65]. McCarty and coworkers found that the right timing of vector delivery after mannitol infusion is crucial: The transduction of vectors can be up to 10-fold more effective when administered 8 min after mannitol pre-treatment compared to 5 min or 10 min in mouse models [152].

SV40 vector was used in therapy for ALS with mannitol pretreatment, to transduce microglia in the CNS with higher efficiency and less systemic exposure [84].

The other BBB opening method is the magnetic resonance-guided focused ultrasound (FUS) with IV-administered microbubbles that can be used for more localized and concentrated vector transduction [158–160]. For more information about FUS and microbubbles, see Ultrasound and microbubbles paragraph.

*Peptide vectors and shuttles.* First, IgG was studied as a peptide shuttle, targeted against insulin and transferrin receptors [161] but was found to be unsuccessful due to its high affinity, which decreased the efficiency of the drug release [162,163]. Therefore, a variety of protein and peptide shuttles have been investigated, most of them are ligands of receptors on the brain endothelium or bioengineered designer peptides.

HIV virus trans-activator of transcription (TAT) peptide is the most used brain delivery peptide shuttle with high permeability across cell membranes [164]. The advantage of this shuttle is also its disadvantage; the easy penetration into any kind of cells makes it lacking brain selectivity. This problem seems to be solvable with combining TAT with higher selectivity receptor ligands, such as T7 peptide or transferrin (Tf) [160]. TAT is transported into the CNS by adsorptive-mediated transcytosis (AMT). AMT means all non-specific vesicular transport mechanisms that do not involve protein receptors. It is generally promoted by the interaction [165].

Cationized albumin was the first studied BBB shuttle [166]. As a drug delivery system, NP have many advantages but also some disadvantage too: It is an endogenous molecule, therefore, it is ensured that it has low toxicity. However, albumin has no brain specificity, but with different proteins conjugated to its surface the delivery through the BBB can be increased as well as its cell specificity, such as Apo E [167] or transferrin [168] specificity. But albumin also has the risk of transmitting blood-borne viral infection if it is derived from human blood, such as Abraxane do, so more attention is needed to produce this type of NP [84].

RVG29 was found during the study of neurotropism of the rabies virus, which is mediated by its glycoprotein (RVG) [169]. The unmodified peptide was shown to reach the brain. It was proved to be a potential gene delivery peptide shuttle: RVG transports oligonucleotides successfully into the brain to silence green fluorescence protein (GFP) in GFP-transgenic mice. RVG29 peptide shuttle uses nicotinic acetylcholine receptor to get through the BBB [170].

Low-density lipoprotein receptors (LDLRs) are the most exploited receptors for delivery across the BBB using peptides, because some of them are more expressed in the brain than in other organs [171] and overexpressed in some tumors [172]. LDLR targeting natural protein ligands, such as ApoB, ApoE fragments [173,174], and Angiopep-2, are widely used as a drug delivery peptide shuttle [170].

Angiopep-2 was discovered when the sequence of aprotinin was compared to other human proteins with Kunitz domain (that interacts with LRP1) with sequence alignment [175]. Angiopep-2 has been used to transport a wide variety of nanocarriers loaded with small molecules, proteins or genetic material into the CNS and is currently in clinical phase study as various types of anti-tumor medicines [170]. Apolipoprotein B100 (ApoB) is the main component of low-density lipoprotein, and it is a ligand of LRP2 [171]. Peptides that derived from ApoE showed higher efficiency in the transportation of other proteins [174,176] because ApoE binds to a variety of LDLRs, including LRP1 [171]. These shuttles are transported by receptor-mediated transport (RMT). As for LDL receptors, LRP1 transporter mediates Angiopep-2 and ApoE, LRP2 and LDLR transport both ApoB and ApoE [170]. Glutathione (GSH), an endogenous peptide, was also investigated as a BBB shuttle after it was reported to be able to cross the BBB. There are many transporters in the CNS [177], that transport GSH and its conjugates [178].

*Designed peptide shuttles.* THR (named after the first three letters of its amino acid sequence) is obtained by phage display that interacts with transferrin receptor (TfR) but does not compete with Tf [179]. THR was successfully increased the transport of gold NPs coated with a peptide (LPFFD) and

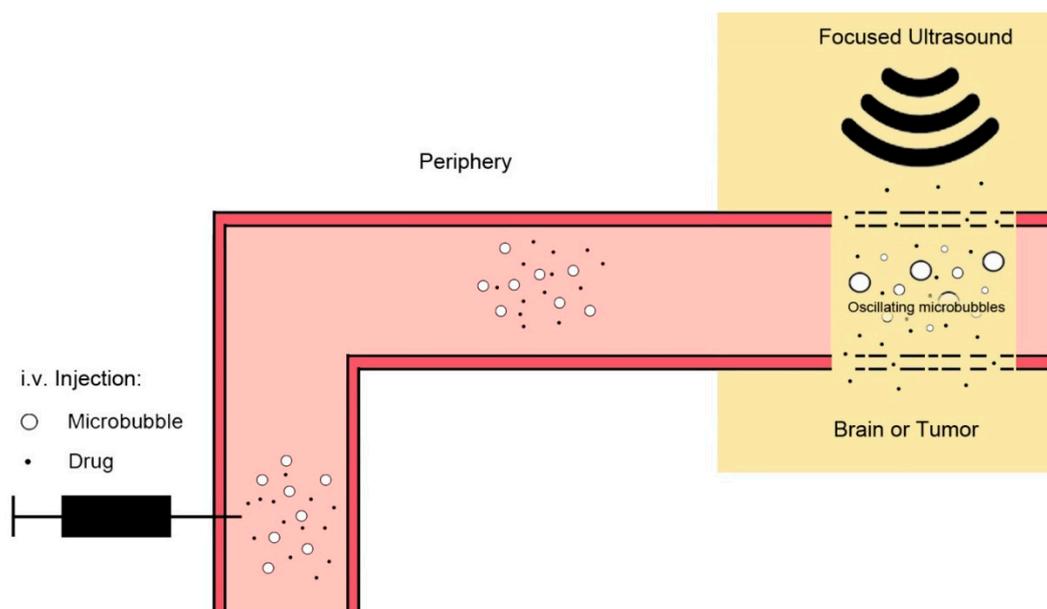
was capable to bind amyloid- $\beta$  and disrupt aggregates with the use of microwave irradiation [180]. THR also directly binds to AAV8 viral vector and facilitates its crossing through the BBB [181].

Phage display was also used to create Peptide-22 specific to LDLR and was tested to deliver chemotherapy for brain glioma treatment [182].

There are some designer BBB shuttles for small drugs (less than 300 Da), that consist of 2 to 4 amino acids and are able to cross the BBB through passive diffusion [170]. A big advantage of these small shuttles is that they minimize the loss of drug effectivity upon conjugation. Some examples are diketopiperazines [183], N-methylphenylalanines [184,185], and phenylprolines [186].

#### 4.2. Ultrasound and Microbubbles

BBB can be disrupted with focused ultrasound (FUS) (Figure 8), but for more location specific drug delivery is combined with intravenously injected microbubbles (MBs), by widening interendothelial clefts and tight-junctions [187]. This method, also called as sonoporation, relies on the mechanical action of the gas MBs in ultrasonic pressure waves [188]. These MBs are about 1 to 10  $\mu\text{m}$  in diameter with a lipid or protein shell, containing heavy gasses, which can be excreted by exhalation and make MBs more stable [188]. These particles also can be used as a drug delivery system by itself, for example, molecules can be attached to the shell [189–191] and they have also been used to deliver stem cells [192] and viral vectors [158,160,193]. Providing MBs with magnetic coating also can increase the effectivity of drug delivery by keeping them in the target area [194].



**Figure 8.** Sonoporation with Focused Ultrasound (FUS). Co-administration of drug or viral vectors with microbubbles is an effective way to delivery therapy into the brain or tumor. Even the peripheral exposure of the drug/vector can be avoided if they are conjugated onto the shell of the microbubbles. Moreover, magnetic microbubbles can be locked in the target area, maximizing the effectiveness of sonoporation.

Sonopermeation was defined by Snipstad and coworkers as the collective name for pore formation and other additional changes in the BBB permeability due to the microbubbles and ultrasound, such as the opening of tight junctions, stimulated endo- and transcytosis, enhanced perfusion, and stromal alterations [188]. This method has many advantages: 1) It can be focused just on the area of the tumor or diseased parts of the brain; 2) the poration does not require high energy ultrasound, therefore, does not cause damage in intermediary tissues; 3) however, the ultrasound can cause some overheating, just enough to increase blood flow [187,195], vascular permeability [196,197] that also modifies the

effectiveness of drug delivery [198–201] in the target area, and 4) membrane integrity regenerates fast (within hours) after the intervention [202]. However, sonopermeation technique can cause some inflammation in the targeted area [203,204], but does not cause ischemia, neither apoptosis and does not damage neurons [205].

#### 4.3. Intranasal Drug Delivery

Intranasally administered drugs can infiltrate the CNS through the olfactory or trigeminal routes by intracellular and extracellular pathways. In the intracellular route, the drug is taken up by olfactory or trigeminal sensory neurons which transmit to the olfactory bulb or to the pons. The extracellular route is between the supporting cells, where the drug is passing through the TJs, paracellular cleft, the lamina propria, perineural space, and then arrives at the subarachnoid space. Through the respiratory route, the drug molecules can reach the brainstem [206,207]. Intranasally administered drugs are distributed mainly in the distal areas of the CNS, besides the olfactory bulb or in the brainstem.

#### 4.4. Receptor-Mediated Opening

Adenosine receptor (AR) substrates can modulate BBB permeability [208]. They can be used for enhanced drug delivery into the brain by activating A2A receptors or restricting the entry of neurotoxic agents or inflammatory immune cells into the brain [209]. This can occur in case of some diseases, such as stroke or sclerosis multiplex, by inhibiting AR activation [210].

Glutamate receptors can also modify the permeability of the BBB; N-methyl-D-aspartate (NMDA) receptor antagonists decrease the permeability. In addition, neuronal activation, using high-intensity magnetic stimulation, increases barrier permeability and facilitates drug delivery [211].

#### 4.5. Efflux Transporter Inhibition

Inhibition of efflux transporters at the BBB can enhance the brain concentration of the substrate drugs which might lead to unwanted and serious CNS side effects from the compounds and also in other cases can provide a therapeutic advantage. However, the delivery of an anticonvulsant, encapsulated carbamazepine NPs was not affected by the inhibition of P-gp, because NPs circumvented the transporters of the BBB [212].

The CNS protective effect of BBB makes it quite difficult to treat brain malignancies or brain metastases, whereas the peripheral diseases are well controlled [213]. The CNS barrier can be partially overcome in the case of efflux transporter substrates by modulation of transporter proteins (e.g., P-gp or Mdr1).

A large number of animal studies, mostly working with Mdr1a/1b(–) knockout mice or dog breeds naturally lacking Mdr1 (e.g., Collie), concluded that the CNS:plasma ratio of the tested anticancer and antiretroviral drugs as well as the opioid loperamide or the calcium channel blocker verapamil can be increased up to 11-, 30-, 20-, and 9-fold, respectively [214–216]. These observations as well as many others which apply substrate-inhibitor combinations [64,65] lead to the conclusion that P-gp has a vital role in preventing the entry of drugs into the CNS. Multidrug resistance proteins (MRPs) and breast cancer resistance protein (BCRP) were also detected on brain endothelial cells [217] and in human brain microvessels [218], respectively. The possibility of large clinical CNS drug interactions was raised under conditions when both multidrug resistance protein 1 (MDR1) and BCRP are inhibited. The phenomenon called ‘P-gp and BCRP synergy’ was described after unexpectedly high CNS accumulation of lapatinib, which was detected in Mdr1a/1b(–/–)–Bcrp double knockout mice, as compared with in the Mdr1a/1b(–/–) or Bcrp single knockouts [219]. The one order of magnitude higher exposure instead of the mathematical sum of the individual increases was later explained by Zamek–Gliszczynski and coworkers [220], namely that brain distribution of such drugs increases asymptotically as a function of the fraction excreted ( $f_e$ ), thus, the increase in exposure is not an additive parameter but a nonlinear function of  $f_e$ . Although the CNS distribution of dual substrates

can be very high in Mdr1a/1b(−/−)—Bcrp double knockout animals, the phenomenon can only occur at complete silencing of both efflux pumps.

#### 4.6. Direct Central Delivery

Unfortunately for these days the invasive route is still the most efficient way to deliver potent drugs into the CNS in sufficient amount without too much peripheral exposure, which can cause toxic side effects. Intracerebral implantation is a traumatic procedure but in severe cases of chronic pain it has been utilized in a number of clinical trials. The implant consists of a biodegradable polymeric matrix or reservoir that contains the therapeutic agents [221]. The advantages of this procedure are sustained and controllable drug release. The skull has to be opened just when the reservoir is implanted, which is fully biocompatible and also the desired location of delivery can be controlled (where the implant is placed). However, the penetration of the drug is strongly dependent on the physicochemical characteristics of the drug, and the implant only can deliver a limited amount of the molecule (reload may be needed).

For example, BCNU (bis-chloroethylnitrosourea) (carmustine)-contained polyanhydride polymer wafer is used to treat recurrent high-grade gliomas [222]. With this matrix a 2-months-long drug release is possible. However, there is an increased risk of trauma and the delivery system is not that effective to warrant this price [223–225].

*Intraparenchymal injection of viral vectors.* For intraparenchymal injection of viral vectors it has been shown that all serotypes of AAV are capable of transducing expression in brain cells of various animals [226–229]. Furthermore, the different serotypes have preferences in cell types: AAV4 primarily targets ependymal cells [230], AAV8 transduces astrocytes, microglia, and oligodendrocytes the most, and AAV9 had the greatest tropism for neurons [226].

*Intraventricular/intrathecal/interstitial delivery.* The most direct route to the CNS is to inject the drug into the intraventricular, intracavitary, or interstitial system. For example, intracerebroventricular (ICV) administration is considered as an intrathecal delivery via an ICV port implanted under the scalp of the patient and is used for treating various types of CNS diseases, such as infections [231,232], chronic pain [233,234], and types of brain cancer [235]. This local delivery ensures high drug concentrations in the CNS while the peripheral exposure is minimal. Direct injections can provide a sufficient dose to treat, for example, primary brain tumors. Such drugs are nitrosourea and methotrexate, and have been used in various clinical trials with promising results. However, being invasive methods have the risk of infection [236].

*Biological tissue delivery.* The idea behind this technique is to implant fetal neural grafts into the dysfunctional area of the brain. This method was used in the treatment of Parkinson's disease [237]. However, these grafts do not survive for long due to a lack of neovascular innervation, but can be elongated with the proper technique of culturing distinct cell types [238].

## 5. Summary

The central nervous system is the most complex organ of the body. It directs our communication with the external world, what we do to our surroundings through our behaviors and what we perceive of our surroundings through our senses. The brain monitors and also controls our internal world, maintaining the balance among our internal organs that are necessary for our sustained life. Any of these functions may be affected by disease or injury. In unbalanced cases, it becomes necessary to consider how and where to deliver drugs to restore or improve the human condition. Our article attempted to summarize the various options to help drug delivery to the brain, to open the BBB or to make some tricks to overcome the barrier. To realize the theory behind the drug delivery concept, it is essential to account for the diverse structure, function, and physiology of the cerebral microvessels and the BBB.

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