Chapter 7
Peptides and Their Delivery to the Brain

Waleed Elballa, Kelly Schwinghamer, Eric Ebert, and Teruna J. Siahaan

Contents

Abstract There has been great progress in utilizing peptides as therapeutic agents in clinical settings. More than 500 peptides are being investigated in preclinical studies; however, there are only a small number of peptides being used for brain diseases. This is due to the difficulty of delivering peptides to the brain. Therefore, this chapter describes the progress of developing methods to deliver peptides to the brain. Several different pathways that are used by peptides to enter the brain from the bloodstream are discussed. Various methods and factors that have been explored for improving the delivery of peptides into the brain are reviewed here.

W. Elballa · K. Schwinghamer · E. Ebert · T. J. Siahaan (✉)
Department of Pharmaceutical Chemistry, School of Pharmacy, The University of Kansas, Kansas, USA
e-mail: welballa@ku.edu; kschwin2@ku.edu; eric.ebert@ku.edu; siahaan@ku.edu

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7.1 Introduction

In the past decades, there have been many advancements in developing peptides as therapeutic and diagnostic agents (Fosgerau and Hoffmann 2015; Kaspar and Reichert 2013; Uhlig et al. 2014) (Fosgerau and Hoffmann 2015). With increasing knowledge to stabilize, formulate, and deliver peptides, the number of approved peptide drugs is expected to increase in the future. Examples of peptide drugs on the market include octreotide, exenatide, integrilin, calcitonin, oxytocin, insulin, and many others. Some of these peptide drugs were derived from natural substances or designed from the active region(s) of proteins.

Many peptides have been developed to treat patients with brain diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS), and brain tumors (e.g., glioblastoma, medulloblastoma) (Oller-Salvia et al. 2016). Unfortunately, the progress in developing peptides for treating brain diseases is still very limited. One of the potential reasons for the lack of progress is the difficulty in effectively delivering peptides into the brain. One of the major challenges to deliver peptides to the brain is their inability to cross the blood-brain barrier (BBB) (Fig. 7.1). In general, the physiochemical properties of peptides (i.e., size, hydrogen bonding potential, cLogP) prevent their passage across the BBB in therapeutically relevant amounts. In this case, most hydrophilic and charged peptides cannot passively diffuse through the membranes of the BBB endothelial cells into the brain (Fig. 7.1; Path A). Some peptides can cross the BBB due to transporters that carry them across the endothelial cells from the systemic circulation stream into the brain (Fig. 7.1; Path B). Many methods have been investigated to improve the delivery of peptide drugs into the brain, including intranasal brain delivery, modulation of the BBB (e.g., osmotic, ultrasound, and adhesion peptides), receptor-mediated endocytosis, and cell-penetrating peptide methods.

Besides the selectivity and potency of peptide drugs to the target receptors, peptide plasma stability and clearance from the systemic circulation are important factors to consider when developing peptide drugs. Peptides are susceptible to enzymatic degradation by exo- and endo-peptidases in the blood. Peptides can also be cleared by the kidney and the liver from the bloodstream. It has been shown that cyclic peptides have higher stability against peptidases in the bloodstream than their respective linear peptides (McCully et al. 2018). The formation of cyclic peptides has been shown to improve peptide permeation through the biological barriers (e.g., intestinal mucosa barrier and the BBB). The incorporation of D-amino acid into the

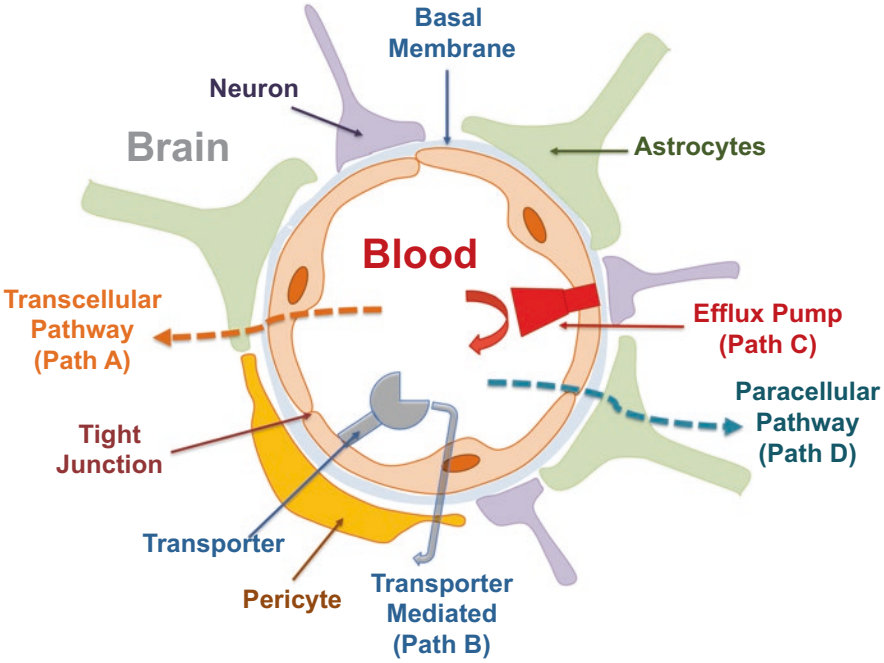


Fig. 7.1 The blood-brain barrier (BBB) is constructed by vascular endothelial cells on the basement membrane, and the cells are connected by the cell-cell adhesion proteins in the intercellular junction to form a tight junction, adherens junction, and desmosome. The vascular endothelial cells are surrounded by astrocytes, neurons, and pericytes. The BBB has transporters (e.g., glucose and amino acid transporter) and efflux pumps (e.g., P-glycoprotein (P-gp)). Peptide drugs can cross the BBB via passive diffusion through the transcellular pathway or paracellular pathway. The transcellular pathway is the diffusion pathway of the peptide through the cell membranes of the BBB, while the paracellular pathway is the diffusion pathway of the peptide between the cells or through the intercellular junctions

peptide could improve plasma stability; this is because proteolytic enzymes do not recognize D-amino acids (McCully et al. 2018). Another method to improve stability without eliminating biological activity is by forming the retro-inverso peptide in which the parent sequence is reversed and all the L-amino acids are replaced with D-amino acids (Chorev et al. 1979; McCully et al. 2018; Ghosh et al. 2010). The rationale is reversing the sequence, and changing the chirality of each amino acid will result in the same presentation of all amino acid side chains on the space for recognition by the target protein or receptor. In addition, the presence of D-amino acid will not be recognized by exo- and endo-peptidases for degradation in the bloodstream and tissues. However, forming the retro-inverso peptide may lower its biological activity because forming the retro-inverso may not mimic the secondary structure of the parent peptide (Li et al. 2010). Furthermore, the reverse peptide

bond presentation in the retro-inverso peptide may not mimic the backbone hydrogen bonding connections between the parent peptide backbone and the receptor; this results in a lower binding affinity of retro-inverso peptide to the receptor compared to the parent peptide (Li et al. 2010).

7.2 Structure of the BBB and In Vitro and In Vivo Models for Brain Delivery

To understand how peptides can enter the brain from the systemic circulation, it is necessary to discuss the structure of the BBB (Fig. 7.1). The BBB is made of endothelial microvessel cells that separate the blood stream and the extracellular fluid of the brain. The BBB endothelial cells wrap around forming tube-like structure with their intercellular cell membranes "glued" to each other by cell-cell adhesion proteins (i.e., occludins, claudins, cadherins, nectins). The abluminal side of the capillary basement membrane of the endothelial cells is populated by extracellular matrix proteins (e.g., collagen) and surrounded by pericytes, neurons, and astrocyte end-feet that anchor the BBB endothelial cells. The BBB has tight junctions in the intercellular space. The BBB endothelial cells are more restrictive compared to those vasculatures found in other parts of the body (Loscher and Potschka 2005; Sharif et al. 2018). The space between the adjacent endothelial cell plasma membranes is referred to as the intercellular junction where small molecules can penetrate through this space as the paracellular transport pathway (Fig. 7.1; Path D). The BBB is distinct from other peripheral capillaries because the BBB endothelial cells are continuous with lacking fenestration as well as having low pinocytosis activity (Loscher and Potschka 2005; Sharif et al. 2018).

The BBB endothelial surface is decorated with receptors, transporters, efflux pumps, and metabolic enzymes. The transporters have a role in carrying nutrient molecules (e.g., glucose, amino acid) into the brain, while the efflux pumps and metabolic enzymes prevent molecules from entering the brain. The efflux pumps include P-glycoprotein (Pgp), breast cancer resistance protein (BCRP), and multidrug resistance-associated protein (MRP) (Hermann and Bassetti 2007; On and Miller 2014). The enzymes metabolize molecules on the surface as well as the ones that transcellularly cross the endothelial cells of the BBB. The metabolic products of the brain are also transported across the BBB into the systemic circulation for their clearance from the brain. The enzymes in the luminal and abluminal membranes of the BBB are gamma-glutamyl transpeptidase, amino acid transport system A 5′-nucleotidase, alkaline phosphatase, and Na PLUS_SPI /K PLUS_SPI ATPase (Sanchez del Pino et al. 1995). Drug molecules that cross the BBB endothelial cells are subjected to metabolism by cytochrome P450 (CYP 450) enzymes (i.e., CYP1B1, CYP3AF, CYP2U1), and this metabolism prevents the intact molecule to cross the BBB (Ghersi-Egea et al. 1995; Shawahna et al. 2011; Ghosh et al. 2011; Dauchy et al. 2008). CYP3A4 oxidizes various molecules while CYP1B1

metabolizes fatty acids (Shawahna et al. 2011; Ghosh et al. 2011). The activity of CYP 450 is also affected by different brain diseases (Kadry et al. 2020).

7.2.1 Method for Assessing BBB Permeability

To simplify transport study of molecules across the BBB, various in vitro cell culture models were developed. Initially, primary culture of bovine brain microvessel endothelial cells was used as a monolayer on a Transwell™ as a model of the BBB (Shah et al. 1989). Unfortunately, the primary culture of BBB was leaky paracellularly with low trans-endothelial electrical resistance (TEER) values with limited uses for studying paracellular transports of molecules. Thus, the in vitro culture model of BBB is not suitable to study transport properties of small molecules including small peptides with up to six amino acid residues; however, this model can be used to predict the transport properties of large peptides and proteins. To improve the BBB tightness, the primary cultures of BBB endothelial cells were co-cultured with pericytes, astrocytes, glial, and neuron cells to mimic in vivo conditions (Fletcher and Callanan 2012; Lippmann et al. 2013). Currently, many immortalized BBB endothelial cell lines have been developed as BBB in vitro models, including murine cells (e.g., bEnd.3.5, TM-BBB), rat cells (e.g., TR-BBB, RBE4), and human cells (e.g., hCMEC/D3, HMEC-1). Recently, 3D models of the BBB have been developed to replicate the in vivo conditions with the BBB endothelial cells surrounded by pericytes, astrocytes, and extracellular matrix proteins. These 3D models have very tight intercellular junctions with TEER values as high as 1650 ohm/cm^2, similar to values found in vivo (Lippmann et al. 2013; Naik and Cucullo 2012; Weksler et al. 2013).

Initially, in situ rat brain perfusion developed by Takasato et al. was used to study the transport of radioactive-labeled (i.e., ^{3}H and ^{14}C) peptides and proteins across the BBB (Takasato et al. 1984; Kiptoo et al. 2011). In this study, the rats undergo surgery, under anesthesia, where a polyethylene catheter filled with heparinized saline is ligated to the left common carotid artery (LCCA). The radioactive-labeled peptide is infused through the LCCA using in perfusate solution delivered by syringe pump immediately after a heart vessel is cut to sacrifice the anesthetized animal. The level of radioactivity in the brain is counted using a scintillation counter to calculate the concentration of peptides delivered to the brain. Brain extraction and LC-MS/MS methods have been developed as alternative techniques to detect deposition of unlabeled peptide in the brain (Ulapane et al. 2017).

Several in vivo methods are also being used to study transport of peptides across the BBB. Intravenous (i.v.) administration via the tail vein followed by detection of peptide deposition in the brain is normally used to study the delivery of molecules across the BBB in animal models. Alternatively, intracarotid artery administration has been used to deliver the peptides, because it has immediate access to the brain vasculature. Recently, IRdye-800 CW-labeled molecules (i.e., peptides and proteins) have been used to determine peptide brain delivery that can be detected using

near IR fluorescence (NIRF) imaging (Ulapane et al. 2017, 2019a, b). The advantage of using NIRF for imaging the deposition of molecules in the brain is that NIRF imaging has a low background interference from the tissues to the emission of light from the analyte molecules. This method can quantitatively determine the amounts of peptides deposited in the brain as well as be sensitive and convenient to study delivery of peptides and proteins into the brain (Ulapane et al. 2017, 2019a, b). Peptides or proteins conjugated to gadopentetic acid (Gd-DTPA) have also been used to study peptide and protein depositions in the brain of living animal in qualitative and quantitative manners (Ulapane et al. 2017; On et al. 2014; Tabanor et al. 2016). The advantage of MRI is that the deposition of the delivered molecule in different sections of the brain can be determined in living animal. In contrast, quantitative determinations of depositions in different brain sections using NIRF have to be done using the dissected and homogenized brain sections. In addition, the access to an MRI instrument may be more limited to majority of researchers compared to that of an NIRF imaging instrument.

7.3 Passive Diffusion Across the BBB via Transcellular Pathway

One way that peptides could cross the BBB endothelium is via the transcellular pathway in which the peptides from the blood partition into luminal cell membranes on the blood side followed by entering the cytoplasm (Fig. 7.1; Path A). From the cytoplasm, the peptide needs to cross the abluminal cell membranes on the brain side to enter the brain extracellular fluid. Normally, molecules with physicochemical properties that follow Lipinski's Rule of Five can diffuse passively via the transcellular pathway of the BBB. To follow the Rule of Five, a peptide molecule should have (a) cLogP lower than 5, (b) MW less than 500 g/mol, (c) less than 5 H-bond donors (e.g., NH, OH), and (d) less than 10 H-bond acceptors (e.g., O and N) (Lipinski et al. 2001; Lipinski 2004, 2016). Most peptides (i.e., hexapeptide or larger) have high hydrophilicity and MW higher than 500 g/mol with more than 5 H-bond donors and 10 H-bond acceptors as well. Thus, these peptides have difficulty in effectively crossing the BBB via passive diffusion. Some peptides and other hydrophilic molecules that have their own transporters can cross the BBB through transcellular pathway into the brain (Fig. 7.1; Path B). For example, the surface of the BBB has transporters for glucose, amino acid, and di-/tri-peptides to carry them from the blood into the brain. These transporters have also been exploited to carry drugs into the brain.

There are molecules with physicochemical properties that follow Lipinski's Rule of Five but still cannot cross the BBB. Molecules belonging to this category are recognized by the efflux pumps such as P-glycoprotein (Pgp) and multidrug resistance (MDR) that expel them from the luminal membranes to prevent them from crossing the BBB (Fig. 7.1; Path C). For example, the anticancer drug daunomycin

has physicochemical properties that are conducive for passive diffusion across the BBB. However, ^{3}H-daunomycin could not effectively cross the BBB when studied using the in situ rat brain perfusion method (Kiptoo et al. 2011). This is because ^{3}H-daunomycin is a substrate for efflux pumps such as P-glycoprotein (Pgp). The recognition of ^{3}H-daunomycin by Pgp can be determined by competition studies using verapamil as an inhibitor of Pgp. Delivering ^{3}H-daunomycin in the presence of verapamil enhanced the brain deposition of ^{3}H-daunomycin (Kiptoo et al. 2011). Thus, ^{3}H-daunomycin is also substrate for Pgp in the BBB (Kiptoo et al. 2011). Some efforts have been made to utilize Pgp inhibitors to improve brain delivery of drugs.

It has been shown that some peptides are substrates for Pgp or MDR on the BBB. Using cell culture models of biological barriers (i.e., intestinal mucosa and BBB), the efflux substrate activity for peptides can be evaluated (Fig. 7.1; Path C) (Ouyang et al. 2002, 2009a, b). If a peptide is a substrate for efflux pumps, the apparent permeability of the substrate of apical (AP) side-to-basolateral (BL) side (AP-to-BL) will be lower than that of BL-to-AP (Ouyang et al. 2009a; On and Miller 2014). This is due to the high presence of efflux pumps on the AP surface of BBB endothelial cells. In other words, the apparent permeability of BL-to-AP is normally larger than that of AP-to-BL. Theoretically, without the effects of the efflux pumps, the passive diffusion of BL-to-AP is the same as AP-to-BL (the ratio of BL-to-AP/AP-to-BL is equal to one). As mentioned previously, efflux pump inhibitors such as cyclosporine A and GF120918 can be used to determine whether the peptide is recognized by efflux pumps. In this case, in the presence of cyclosporine A or GF 120918, the AP-to-BL transport across the BBB of the peptide under study increases (On et al. 2014; On and Miller 2014).

The formation of cyclic peptides from the parent linear peptides improves the peptide passive transport by two- to threefold across Caco-2 cell monolayers via the transcellular pathway in the absence of efflux pump activity (Fig. 7.1; Path A) (Okumu et al. 1997). To enhance delivery of peptides, a cyclic peptide prodrug using acyloxyalkoxy promoiety was synthesized to improve the delivery of delta-sleep-inducing peptide (DSIP: H-Trp-Ala-Gly-Gly-Asp-Ala-OH; Fig. 7.2a) (Pauletti et al. 1996). The acyloxyalkoxy cyclic peptide prodrug of DSIP (AOA-DSIP; Fig. 7.2b) has significantly better transport across the Caco-2 cell monolayers than that of the parent linear peptide (Fig. 7.2a) (Pauletti et al. 1996). This indicates that the formation of the cyclic prodrug can enhance the passive diffusion across the cell membranes of Caco-2 cell monolayers. The cyclic prodrug can also be converted to the parent linear peptide by esterase.

The same cyclic peptide prodrug method was also applied to the delta opioid peptide called (D-Ala 2, D-Leu 5) enkephalin (DADLE: H-Tyr-D-Ala-Gly-Phe-D-Leu-OH, Fig. 7.2c). As observed using a model hexapeptide, it was hypothesized that cyclization of DADLE as prodrugs would improve the passive diffusion across the BBB compared to parent linear DADLE peptide. The formation of cyclic peptide prodrugs would lower the hydrogen bonding potential and enhance partitioning to cell membranes. Thus, several different cyclic prodrugs of DADLE were synthesized, including acyloxyalkoxy-based cyclic prodrug of DADLE (AOA-DADLE;

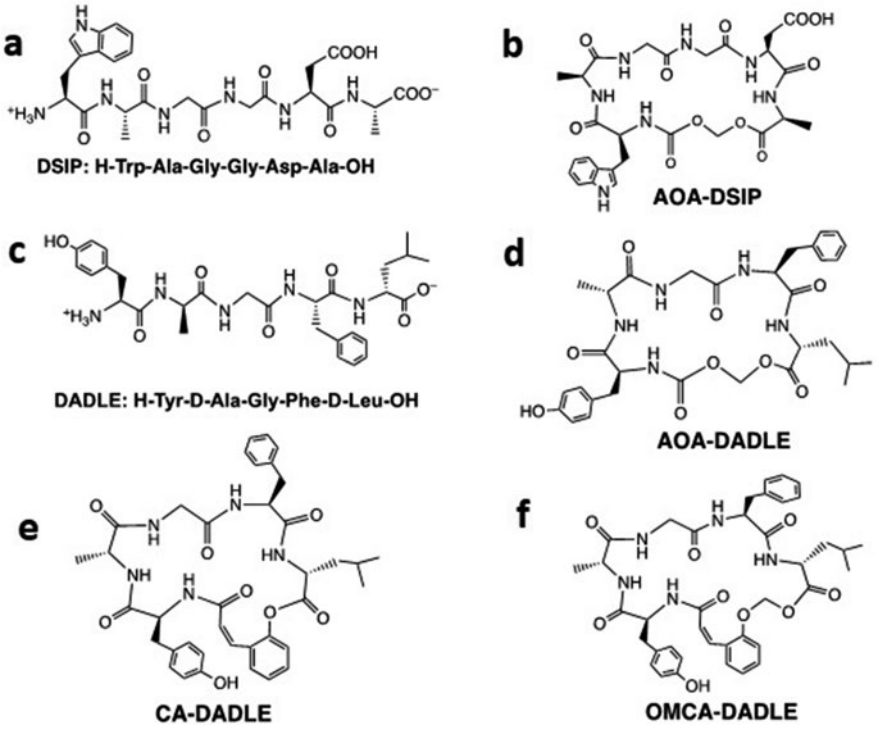


Fig. 7.2 (**a**–**f**) Structure of DSIP and DADLE peptide and their cyclic prodrugs. The cyclic peptide prodrugs (**b** and **d**–**f**) were synthesized to improve permeation of the peptide across the biological barriers such as the BBB and intestinal mucosa barrier by changing their physicochemical properties to become conducive the partition to cell membranes of the biological barriers. The cyclic peptide prodrugs were constructed using various esterase-sensitive promoieties such as acyloxyalkoxy (AOA, **b** and **d**), coumarinic acid (CA, **e**), and oxymethyl-modified coumarinic acid (OMCA, **f**) promoiety. The peptide prodrugs can be converted to parent peptides by esterase in the blood and brain

Fig. 7.2d), coumarinic acid-based cyclic prodrug of DADLE (CA-DADLE; Fig. 7.2e), and oxymethyl-modified coumarinic acid-based cyclic prodrug of DADLE (OMCA-DADLE; Fig. 7.2f) (Ouyang et al. 2002, 2009a). Although the membrane partition and passive diffusion of these cyclic peptide prodrugs were improved compared to parent linear peptides, the cyclic peptide prodrugs of DADLE are substrates of Pgp efflux pump. Therefore, the overall penetration across biological barriers was impeded by Pgp (Fig. 7.1; Path C). The recognition of cyclic prodrug by efflux pump was determined using various cell monolayers such as Caco-2, MDCK-WT, MDCK-MDR1 (Pgp), and MDCK-MRP2 cells. MDCK-WT, MDCK-MDR1, and MDCK-MRP2 cell monolayers have been used as alternative models for the BBB. MDCK-MDR1 and MDCK-MRP2 cells have high expression of MDR1 and MRP2 efflux pumps on their surface, respectively, to determine cyclic peptide prodrug recognition by the efflux pumps. In general, the data showed that

AOA-DADLE, CA-DADLE, and OMCA-DADLE have higher BL-to-AP transport than AP-to-BL transport, indicating that all three cyclic peptides are substrates for efflux pumps (Ouyang et al. 2002, 2009a). The ratios of BL-to-AP/AP-to-BL for AOA-DADLE, CA-DADLE, and OMCA-DADLE were 16, 35, and 35, respectively (Ouyang et al. 2002, 2009a). Transport studies of AOA-DADLE, CA-DADLE, and OMCA-DADLE were also done in the presence of efflux pump substrate (Pgp inhibitors) such as GF 120918, cyclosporin A, and PSC-833. The data indicated that the BL-to-AP/AP-to-BL ratios for AOA-DADLE, CA-DADLE, and OMCA-DADLE when delivered with GF 120918 were lowered to 0.5, 1.02, and 1.48, respectively (Ouyang et al. 2002, 2009a). This is an indication that all three cyclic prodrugs are substrates for efflux pumps. Using in situ rat brain perfusion method, the GF-120918 enhanced the delivery of CA-DADLE into the brain by 460-fold compared to that of CA-DADLE alone (Ouyang et al. 2009a). This is another confirmation that CA-DADLE is a substrate for Pgp efflux pump on the rat BBB. Unlike small molecules in which structural modification can avoid the efflux pump recognition to improve passive permeability, the structural change in peptides may not significantly improve their passive diffusion because the changes cannot overcome the efflux pump activity.

7.4 Receptor-Mediated Transcytosis of Peptides Through the BBB

The BBB is decorated with many transporters and receptors that can carry molecules from the blood stream into the brain (Fig. 7.1; Path B). These transporters include glucose, amino acid, and di-/tripeptide transporters. Glucose transporter, GLUT-1, is a saturable and efficient transporter to provide a glucose for the brain to function properly. This transporter facilitates glucose diffusion to create balance from the higher to the lower concentration between the blood and the brain; thus, any excess and unutilized glucose in the brain is transported back into the blood (Banks et al. 2012). The brain metabolic rate can be measured by determining the rate of glucose uptake (Banks et al. 2012). GLUT-1 has also been exploited for drug delivery into the brain; however, the success of this method has not yet been realized.

Similarly, there are many protein transporters on the surface of the BBB endothelial cells, including transporters for insulin (Frank and Pardridge 1981; Frank et al. 1986), insulin-like growth factor 1 (IGF-1) (Frank et al. 1986), and transferrin (Tf) (Fishman et al. 1987; Visser et al. 2004) melanotransferrin (p97) (Demeule et al. 2002), apolipoproteins (Apo) A and E (Herz and Marschang 2003), leptin (Banks and Farrell 2003), immunoglobulin G (Zlokovic et al. 1990), tumor necrosis factor alpha (TNF-α) (Pan and Kastin 2002), epidermal growth factor (Pan and Kastin 1999), and interleukin (Banks et al. 2001). As an example, insulin is transported by insulin receptor across the BBB into the brain via a saturable mechanism (Banks 2004). The insulin transporter regulates the balance between insulin in the blood

and the brain. The balance is also affected by glucose in which during a hyperglycemic condition, the transport of insulin across the BBB is increased.

The use of receptor-mediated transport system normally relies on a higher expression of receptors on the luminal side of the BBB compared to those in other organs. Therefore, the ligand is targeted to the BBB endothelial cell rather than other organs (Oller-Salvia et al. 2016; Broadwell et al. 1988; Frank et al. 1986). Thus, protein or peptide ligands can be conjugated with drugs to make protein-drug or peptide-drug conjugates for targeting drugs into the brain. In some cases, the receptor-mediated uptake of the conjugate can be inhibited by the endogenous ligand (e.g., peptide, protein), which lowers transport effectiveness of the conjugate to cross the BBB (Oller-Salvia et al. 2016). To avoid competition between the conjugate and endogenous ligand for the receptor, monoclonal antibodies (mAbs) or peptide ligands that bind to the transport receptor at a different site from that of endogenous ligand were developed. In this case, the mAb or the targeting peptide can avoid interrupting the receptor function to bind its endogenous ligand.

Several mAbs have been developed as antibody-drug conjugates (ADCs) to carry drugs across the BBB into the brain. MAbs to transferrin and insulin receptors have been developed as ADCs to deliver drugs into the brain. Transferrin receptor (TfR), found abundantly on the BBB, transfers iron into the cells by transporting iron-bound transferrin (Tf) proteins. Many clinical developments of TfR mAbs as ADC therapeutics have been carried out for brain diseases, where the major challenge was to increase transcytosis efficiency of ADC into the brain (Paterson and Webster 2016). Normally, the uptake of TfR ADC from the luminal side into the BBB endothelial cells is effective; however, the translocation of TfR ADC from the endothelial cells into the brain can be inefficient. This is due to trapping of TfR ADC in the endosomes of endothelial cells; furthermore, tight binding of TfR mAb to TfR causes a high degradation of TfR ADC in lysosomes of endothelial cells. It has been shown that the higher the affinity of the TfR mAb to TfR, the higher the degradation of ADC in lysosomes. This limits the transcytosis of ADC into the abluminal region. Due to the mAb tight binding to TfR, the release of TfR ADC from TfR receptor at the abluminal side of endothelial cells into the brain could also be inefficient. Overall, the TfR ADC released into the brain fluid to diffuse throughout the brain is lower than the uptake from the blood. There have been some efforts to lower the binding affinity of TfR mAb to TfR to improve the transcytosis efficiency of TfR ADC from the blood into the brain. Recently, a phage display method was used to discover new peptide ligands for TfR that can be used for drug delivery across the BBB (Oller-Salvia et al. 2016).

The brain transport mechanisms of opioid peptides such as D-Phe-Cys-Tyr-D--Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP), [D-penicillamin^{2,5}] enkephalin (DPDPE), and biphalin (Tyr-D-Ala-Gly-Phe-NH_2) were compared using in situ rat brain perfusion method (Egleton et al. 1998). It was found that CTAP penetrated the BBB via passive diffusion, while DPDPE crossed the BBB through a combination of passive diffusion and saturable transport mechanism. Finally, biphalin crossed the BBB via passive diffusion as well as utilizing large neutral amino acid carrier (Egleton et al. 1998). Both DPDPE and biphalin showed time-dependent linear uptake, and both

had higher brain uptake than sucrose, while CTAP had higher uptake than inulin (Egleton et al. 1998). To test whether all three peptides were transported by receptor-mediated process, the uptake of radioactive-labeled peptide was inhibited by unlabeled peptide. Addition of 100 μM non-radioactive DPDPE and biphalin peptides inhibited the uptake of both ^{3}H-DPDPE and I^{125}-biphalin, respectively, suggesting both peptides were transported by receptor-mediated process. In contrast, the uptake of ^{3}H-CTAP was not inhibited by non-radioactive CTAP, indicating that the BBB penetration of CTAP was not mediated by receptor (Egleton et al. 1998).

To ensure that the measured peptide radioactivity was due to deposition in the brain and not from the trapping of the peptide in the BBB endothelial cells, a capillary depletion experiment was carried out. The capillary depletion was done to remove the BBB vascular endothelial cells from the brain homogenates. The capillary depletion study showed that only 1% of biphalin and 10% of DPDPE were in the BBB vascular endothelial cells, while the rest of peptide resided in the brain as measured by radioactivity. These data indicated that majority of brain radioactivity came from biphalin and DPDPE that were deposited in the brain parenchyma not in the vasculature (Egleton et al. 1998). In contrast, 50% of CTAP resided in the vasculature, and 50% was distributed in the brain, indicating a high amount of the CTAP was trapped in the BBB vascular endothelium (Egleton et al. 1998). The uptake of both ^{3}H-DPDPE and ^{125}I-biphalin in the rat brain was partially saturable, and they followed Michaelis-Menten kinetics (Egleton et al. 1998). Therefore, it is critical to perform capillary depletion studies to ensure that the detected molecules are in the brain not in the BBB endothelial vasculatures.

The brain uptake of CTAP was studied using ^{3}H-CTAP and compared to ^{14}C-inulin and ^{3}H-morphine using the in situ brain perfusion method (Abbruscato et al. 1997). ^{14}C-inulin is a paracellular marker, and ^{3}H-morphine is a μ-opioid receptor agonist. The radioactivity of ^{3}H-CTAP was six times higher than that of ^{14}C-inulin in the brain and cerebral spinal fluid (CSF) at different time points. However, the brain deposition of ^{3}H-CTAP was lower than ^{3}H-morphine, due to morphine's higher hydrophobicity than CTAP leading to more passive diffusion across the BBB (Abbruscato et al. 1997). CTAP has reasonable stability in the serum and blood with $t_{1/2} > 500$ min. Presumably, CTAP's high binding affinity to albumin contributes to CTAP's plasma stability.

Ghrelin, a 28-mer peptide, can cross the BBB with unelucidated mechanism (van der Lely et al. 2004; Rhea et al. 2018). The BBB transport of ghrelin was proposed to be mediated by the growth hormone secretagogue receptor (GHSR); however, GHSR was not the only receptor that can transport ghrelin into the brain (Rhea et al. 2018). Human and mouse desacyl ghrelins (DAGs) were transported across the BBB faster than acyl ghrelin (AG), indicating that acylation levels of ghrelin influence the brain uptake. Injection of ghrelin peptides via i.v. administration led to a measurable influx into the brain from the blood stream. Human ghrelin peptides are usually transported more efficiently across the BBB than mouse ghrelin; their transport rates are in the following order hDAG > mDAG > hAG > mAG (Rhea et al. 2018). The highest level of ghrelin was found in the olfactory bulb, regardless of their structures (i.e., acylated or non-acylated). Human DAG peptide has the fastest

influx rate into the brain, while mouse AG peptide has the slowest influx rate in both wild-type (WT) and GHSR null mice (Rhea et al. 2018). There was no significant difference of ghrelin influx rate between WT and GHSR null mice, indicating that GHSR was not the main transport receptor (Rhea et al. 2018). Using ^{125}I-hDAG and acid precipitation, hDAG was found to be intact in serum and the brain after crossing the BBB. The brain uptake of ^{125}I-hDAG was not saturable, suggesting the transport was not a receptor-mediated process. After the uptake, ghrelin was distributed throughout the brain with high levels in the olfactory bulb and pons-medulla (Rhea et al. 2018).

Cell-penetrating peptides (CPPs), such as transactivator of transcription (TAT) peptides from fragments of HIV TAT protein, have been shown to undergo adsorptive-mediated transcytosis (AMT) across the BBB (Oller-Salvia et al. 2016; Herve et al. 2008; Green and Loewenstein 1988; Frankel and Pabo 1988). There are over 100 CPPs, from 5-mer to 40-mer peptide, that have been investigated over several decades (Lindgren and Langel 2011). CPPs have been investigated to carry drugs across the BBB into the brain (Oller-Salvia et al. 2016). TAT peptide was used to delivery β-galactosidase into the brain by aiding the transcellular passage of the enzyme across the BBB (Oller-Salvia et al. 2016; Herve et al. 2008). Unlike receptor-mediated process, the cellular uptake of CPP is unsaturable; cells from organs other than BBB endothelial cells also can engulf CPP. In general, cellular uptake of CPPs was not selective to a particular group of cell type, and their exocytosis from the BBB endothelium into the brain needs further investigation. There is a possibility that CPPs and their cargo could be trapped in the BBB endothelium without entering the brain (Oller-Salvia et al. 2016). In summary, BBB selectivity andtranscytosis effectiveness of CPPs across the BBB need further investigation.

7.5 Peptide Conjugates for Delivering Drugs Across the BBB

Several peptides have been found to deliver drugs across the BBB via receptor-mediated process. Angiopep-2 peptide (ANG, Table 7.1) was found to undergo receptor-mediated transcytosis across the BBB endothelial microvessel cells (Li et al. 2016). This peptide was transported by low-density lipoprotein-1 (LRP1) receptors on the surface of the BBB endothelial cells (Li et al. 2016). Conjugation of ANG peptide with β-secretase inhibitor (SI) produced ANG-SI peptide (Table 7.1). ANG-SI inhibited the production of amyloid-beta (Aβ) in neuronal cells, demonstrating that ANG peptide was responsible for the internalization of SI peptide that led to SI peptide activity (Kim et al. 2016).

A combination of ANG and TAT (Table 7.1) peptides were used to deliver paclitaxel (PTX) by forming ANG-TAT-PTX conjugate that treats U87 glioblastoma brain tumors in mice (Li et al. 2016). Mice treated with ANG-TAT-PTX conjugate had higher survival rate than those treated with ANG-PTX conjugate and PTX alone (Li et al. 2016). The results signify that ANG-TAT combination improved targeting

Table 7.1 Peptide sequences for drug delivery

Peptide name	Sequence
ANG	TFFYGGSRGKRNNFKTEEY
ANG-SI	TFFYGGSRGKRNNFK–EVN-sta-VAEF
ANG-TAT	TFFYGGSRGKRNNFK–TEEYGRKKRRQRRRPPQQ
gHo	NHQQQNPHQPPM-NH_2
pVEC	LLIILRRRIRKQAHAHSK-NH_2
GKRK	GKRK
RVG29	YTIWMPENPRPGTPCDIFTNSRGKRASNGC
C2	CDIFTNSRGKRA
C2-9r	CDIFTNSRGKRAGGGGrrrrrrrrr
RI-C2	Arkgrsntfidc
RI-C2-9r	arkgrsntfidcGGGGrrrrrrrrr

of PTX into the brain and brain tumor. Presumably, the brain uptake of ANG-TAT-PTX conjugate was due to recognition of ANG peptide by LRP-1 receptors as well as adsorptive-mediated transcytosis (AMT) of TAT peptide by the BBB endothelial cells. In summary, the combination of ANG and TAT peptide is better than ANG peptide alone in delivering PTX to the brain and brain tumor cells.

Glioma-homing (gHo) peptide (Table 7.1) was discovered using phage display method, because it binds to U251 glioma cells (Eriste et al. 2013). gHo peptide was conjugated to a CPP called pVEC peptide (Table 7.1) to make pVEC-gHo peptide. The combined peptide delivered 5(6)-carboxy fluorescein (FAM) and doxorubicin (Dox) by conjugating the drug to its N-terminus to produce FAM-pVEC-gHo and Dox-pVEC-gHo conjugates, respectively. FAM-pVEC-gHo selectively bound to U87 tumor cells but not HeLa and HEK even at very high concentration as determined by confocal microscopy; this signifies the selectivity of the peptide combination (Eriste et al. 2013). The gHo peptide directs the conjugate (i.e., FAM-pVEC-gHo or Dox-pVEC-gHo) to glioma cells, while pVEC has a role to improve uptake via AMT for transcytosis across the BBB (Eriste et al. 2013). Administration of FAM-pVEC-gHo conjugate to animal with subcutaneous U87 tumors showed deposition of the conjugate in tumor but not in other tissues such as the brain, kidney, and liver. Dox-pVEC-gHo conjugate was used to treat animals with subcutaneous U87 tumor; the tumors in treated mice were significantly smaller than those in untreated group. Unfortunately, both free doxorubicin and Dox-pVEC-gHo derivative did not prolong the survival of animal with intracranial gliomas, indicating that the conjugate did not cross the BBB effectively (Eriste et al. 2013). Further studies are still required to assess whether Dox-pVEC-gHo can cross the BBB to improve the outcome of treating intracranial tumors in mice (Eriste et al. 2013).

7.6 Brain Drug Delivery Using Nanoparticles

Nanoparticles have been investigated to deliver small molecule drugs, peptides, proteins, and oligonucleotides into the brain. A VCR-GKRK-APO nanocage delivery system was developed using a dual targeting system with GKRK peptide and apoferritin (APO) that encapsulates vincristine (VCR) as anticancer drug (Zhai et al. 2018). This nanocage was used to treat glioma brain tumor in the animal model. In this nanocage, GKRK peptide targets heparan sulfate proteoglycan (*HSPG*), while APO is used to target transferrin receptor-1 (TfR1) in the BBB endothelial and tumor cells. HUVEC and U87MG cells internalized fluorescence-labeled GKRK-APO significantly better than that of APO alone, suggesting that a dual targeting system was better than the single targeting system (Zhai et al. 2018). It was also shown that drug-free GKRK-APO and APO were not toxic to HUVEC and U87MG cells as determined by MTT assay, indicating the nontoxic nature of the GKRK-APO drug carrier (Zhai et al. 2018).

The uptake of GKRK-APO nanocages by tumor cells was followed using noninvasive NIR fluorescence (NIRF) imaging and immunofluorescence methods. After administration, a higher accumulation of GKRK-APO nanocages found in glioma brain tumor cells implanted in animals compared to APO nanocages. This demonstrates that GKRK-APO nanocage can cross the BBB to target the tumor cells. Compared to free vincristine alone, vincristine-loaded GKRK-APO has enhanced cytotoxicity against U87MG tumor cells. In addition, it can cross the bEnd3 cell monolayer BBB in vitro model (Zhai et al. 2018). In vivo, the brain deposition of vincristine was 6.5-fold higher when delivered using the GKRK nanocage compared to that of free vincristine (Zhai et al. 2018). The blood clearance of VCR-GKRK-APO was also slower than that of vincristine alone. The animals treated with the VCR-GKRK-APO nanocage have smaller glioma tumor diameter compared to controls as determined by MRI (Zhai et al. 2018). Histology studies indicated no significant damage to the liver, kidney, brain, lung, and heart cells after administration of VCR-GKRK-APO nanocage. The histology studies implied that vincristine delivery with nanocage was not toxic for other non-targeted organs. In summary, nanocages with dual targeting moieties enhanced vincristine brain delivery and selectivity in the animal models.

Another example of improved nanoparticle BBB delivery through peptide targeting involves the rabies virus glycoprotein (RVG) (Kumar et al. 2007; Liu et al. 2009; Javed et al. 2016). A 29-amino acid peptide from rabies virus glycoprotein (RVG) was conjugated via a polyethylene glycol (PEG) linker to polyamidoamine dendrimers (PAMAM) to produce PAMAM-PEG-RVG29 nanoparticles (NPs) for delivering genes into the brain (Liu et al. 2009). Recently, RVG peptide was proposed to bind $GABA_B$ receptor where it was previously proposed to bind nicotinic acetylcholine receptor (nAchR) (Liu et al. 2009). Fluorescently labeled NPs were engulfed by brain capillary endothelial cells (BCEC). To test whether the cellular uptake was via a receptor-mediated process, the NPs were first incubated on BCECs at 4 °C and 37 °C. Lower cellular uptake of NPs was observed at 4 °C compared to

37 °C incubation. Suppression of NP internalization activity at 4 °C implies that the internalization is due to receptor-mediated uptake process. Preincubation of cells with free RVG29 peptide inhibited the uptake of PAMAM-PEG-RVG29 NPs, supporting the idea that the uptake of NPs by BCEC was mediated by receptors of RVG29 peptide. The uptake of NPs was also inhibited by GABA, verifying that the internalization of RVG2-studded NPs was mediated by $GABA_B$ receptors through clathrin-mediated endocytosis (Liu et al. 2009). In contrast, acetylcholine, nicotine, or mecamylamine did not inhibit the uptake of PAMAM-PEG-RVG29 NPs, showing that the nicotinic acetylcholine receptor (nAchR) was not the internalization receptor of the NPs (Liu et al. 2009).

DNA was incorporated into PAMAM-PEG-RVG29 NPs via charge-charge interactions. PAMAM modification with RVG29 peptide did not influence the DNA encapsulation properties compared to unmodified PAMAM. PAMAM-PEG-RVG29 NPs were effective in delivering DNA into cell cytoplasm after incubation for 15 and 60 min, and more DNA was found in the cytoplasm than in the nucleus (Liu et al. 2009). After i.v. administration of fluorescent-labeled PAMAM-PEG-RVG29/DNA NPs, the RVG29 containing NPs accumulated in the brain with a significantly higher amount than that of fluorescent-labeled PAMAM/DNA NPs. The results confirmed the important role of RVG29 peptide in the cellular uptake of NPs (Liu et al. 2009). In addition, delivery of PAMAM-PEG-RVG29/pGL2 increased the transfection efficiency of luciferase in the brains of mice compared to those injected with control PAMAM/pGL2 (Liu et al. 2009).

RVG peptide was also used to deliver siRNA across the BBB for silencing neuronal gene activity (Kumar et al. 2007). RVG peptide bound to green fluorescent protein (GFP)-encoding lentiviral vector could specifically transfect neuronal Neuro-2a cells but not HeLa cells in vitro, indicating cell selective delivery of GFP gene by RVG peptide. RVG peptide bound to anti-GFP siRNA can silence the GFP gene in Neuro-2a cells (Kumar et al. 2007). Similarly, GFP transgenic mice dosed with RVG peptide linked to anti-GFP-siRNA significantly suppressed GFP expression compared to control. There were no significant differences in GFP expression in all other organs compared to control, confirming that RVG directed anti-GFP-siRNA into the brain (Kumar et al. 2007). RVG peptide was also used to target antiviral FvE hairpin RNA in mice infected with Japanese encephalitis virus (JEV). The results showed an increase in survival of infected mice by 80% compared to untreated control mice; the untreated mice all died within 10 days (Javed et al. 2016). These results indicated that RVG peptide transported siRNA across the BBB to silence the gene for JEV replication (Javed et al. 2016).

C2 decapeptide (Table 7.1) was derived from the RVG29 peptide, where it can bind to neuronal cells as well as be transported through the BBB into the brain (Javed et al. 2016). C2 peptide and retro-inverso C2 peptide (RI-C2, Table 7.1) have been utilized to deliver siRNA into the brain. RI-C2 has a reversed sequence of C2 with D-amino acids. M17 cells can uptake complexes of C2-9r and RI-C2-9r peptides with rhodamine-labeled siRNA into the cytoplasm. C2-9r and RI-C2-9r peptides have nine D-arginine residues added to C2 and RI-C2 spaced by four Gly residues to bind siRNA via opposite charge interactions to make C2-9r-siRNA and

RI-C2-9r-siRNA complexes (Javed et al. 2016). Delivery of C2-9r-siRNA complex could knockdown 60–90% of the α-synuclein protein expression. RI-C2-9r-siRNA behaved the same way as the parent C2-9r-siRNA; however, siRNA in the RI-C2-9r-siRNA had longer plasma stability than that in the parent C2-9r-siRNA formulation (Javed et al. 2016). Delivery of C2-9r-siRNA and RI-C2-9r-siRNA via i.v. administration into mice could knockdown α-synuclein levels for up to 72 h in different parts of the brain without eliciting an immune response (Javed et al. 2016). The downregulation of α-synuclein level is protected against neurodegeneration and pathological symptoms in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease (Javed et al. 2016). The treated mice also showed improvement in rotarod behavioral tests with decreased dopaminergic neuronal loss compared to untreated control mice (Javed et al. 2016). The results demonstrated that C2-9r and RI-C2-9r peptides can effectively deliver siRNA into the brain.

Although RVG29 peptide and its derivatives can cross the BBB, further studies will be needed to improve the transport capacity and selectivity to carry molecules across the BBB. In addition, the mechanism of transport of RVG29 and its derivatives is still unclear because some data suggest that the uptake of RVG29 is due to nicotinic acetylcholine or $GABA_B$ receptors rather than RVG receptor. Thus, more studies are needed to elucidate the transport mechanism of RVG29 and its derivatives.

7.7 Modulation of the BBB to Improve Delivery via Paracellular Pathways

Ions and small hydrophilic molecules can penetrate through the BBB via the paracellular pathways (Fig. 7.1; Path D). The paracellular pathways only allow molecules with hydrodynamic radius of <11 Å to cross the BBB because the tight junctions have small pores that limit passive diffusion of many medium (e.g., peptides) or large molecules (e.g., proteins). The BBB intercellular junctions consist of three different sections. First, the tight junction section is found in the luminal side and forms the closest membrane-membrane contact between opposing cells. The tight junctions, normally referred to as the "Kiss" region, provide the most restricted passage way. The glue between the opposing membranes is constructed by protein-protein interactions of occludins, claudins, and junctional adhesion molecules (JAMs). The second section underneath the tight junction is *adherens* junction connected by cell-cell adhesion proteins such as VE-cadherins and nectins. The final section is the desmosome that is constructed by desmocollin and desmoglein interactions where desmocollins and desmogleins are in the classic cadherin family.

Many efforts have been investigated to improve delivery of peptides and proteins into the brain via modulation of paracellular pathways. Most of these methods were aimed at increasing the porosity of the intercellular junctions to enhance passive

diffusion of peptides and proteins from blood to brain via the paracellular pathway (Fig. 7.1; Path D). The most successful method to deliver drugs to brain tumor patients is the blood-brain barrier disruption (BBBD) method utilizing hyperosmotic solution. Several additional methods have been developed to selectively modulate the protein-protein interactions in the intercellular junctions of the BBB using cell adhesion peptides.

7.7.1 Osmotic Blood-Brain Barrier Disruption (BBBD) Method

The BBBD, or osmotic brain delivery method, has been successfully used to deliver anticancer drugs to brain tumor patients. This method utilizes hypertonic mannitol solution to disrupt the BBB to increase the porosities of the BBB intercellular junctions (Neuwelt et al. 1984a, b; c, 1985; Doolittle et al. 2014). In this case, administration of hypertonic solution via internal carotid artery (ICA) shrinks the vascular endothelial cells of the BBB to create large pores in the BBB paracellular pathways to allow passive diffusion of drug molecules (e.g., small drugs and proteins) through the paracellular pathways into the brain (Fig. 7.1; Path D). One caution is that prolonged opening of the BBB by osmotic method may cause brain inflammation and epilepsy (Luo and Shusta 2020; Marchi et al. 2007). Besides modulation of the intercellular junctions, osmotic brain delivery method has also been suggested to induce vesicular transport as well as the presence of fenestrations.

In preclinical studies, the BBBD method was used to infuse radioactive-labeled methotrexate (MTX) into rat with tumor on the right hemisphere at a dose of 4000 ng/g body weight (Neuwelt et al. 1984a). The brain depositions of MTX were compared after infusions of MTX with the same dose without hypertonic mannitol and with hypertonic mannitol into the right ICA of rats. The data showed that infusion of MTX with hypertonic mannitol into the right ICA has higher depositions of MTX in tumor (5×), tissue surrounding tumor in the right hemisphere (2.9×), and brain tissue distant to tumor (10×) compared to infusion of MTX alone (Neuwelt et al. 1984a). This suggests that mannitol disrupts the BBB to allow permeation of MTX into the brain and tumor. However, there was no difference in MTX depositions in contralateral left hemisphere of the brain when MTX was delivered with and without mannitol. The results indicate that infusing MTX with mannitol to the right ICA did not effectively enhance delivery to the left hemisphere of the brain. In other words, the MTX did not effectively diffuse from the right to the left hemisphere of the brain. When MTX was infused to the left ICA with hypertonic mannitol, the deposition of MTX on the left hemisphere was higher than MTX found in tumor on the right hemisphere, area surrounding the tumor, or right brain distant from the tumor. These results indicate that hypertonic solution can enhance the delivery of anticancer drug MTX into the brain and tumor. In addition, the ICA site

chosen for administering the drug can influence the deposition of the drug in the targeted hemisphere of the brain.

7.7.2 *Blood-Brain Barrier Modulators (BBBMs) of the Intercellular Junction Proteins*

Recently, focused ultrasound (FUS) has been developed to modulate the intercellular junctions of the BBB transiently to improve drug permeation from the blood into the brain. The effects of FUS in delivering microbubbles containing drugs across the BBB have been observed using MRI (Burgess et al. 2015). In preclinical study, FUS delivery of etoposide to glioblastoma in mice increased brain tumor-to-blood ratio by 3.5-fold, and it prolonged animal survival and decreased tumor growth by 45% (Wei et al. 2021). Drugs with various sizes and doses in different sizes of microbubbles can be effectively delivered to the brain using FUS with different frequency and repetition of ultrasound pulses in preclinical studies (Burgess et al. 2015). It is envisioned that the noninvasive nature of FUS in combination with MRI monitoring is a clear advantage of this method over invasive brain delivery methods (e.g., intracerebroventricular injection). However, the repeatability of FUS in the clinical setting is still unclear, and the side effects from off-target delivery of FUS to brain tissues require full and extensive investigations.

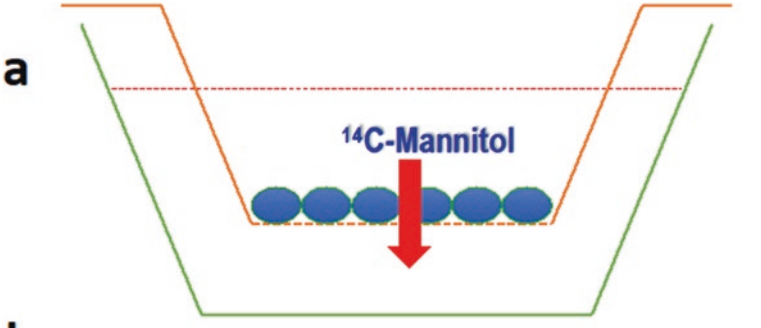
Inspired by the BBBD method using hypertonic mannitol, a new method to modulate the BBB to increase porosity of the paracellular pathway was developed by modulating the cell-cell adhesion molecules connecting the two opposing cell membranes (Lutz and Siahaan 1997b). The idea is that inhibiting cell-cell adhesion molecules can increase the BBB paracellular pathway porosity and enhance the paracellular permeation of molecules across the BBB. Inhibition of

Table 7.2 Sequences of BBBM: HAV and ADT peptides

Peptide name	Sequence
HAV peptides	
HAV6	Ac-SHAVSS-NH_2
HAV4	Ac-SHAVAS-NH_2
cHAVc1	Cyclo(1,8)Ac-CSHAVASC-NH_2
cHAVc3	Cyclo(1,6)Ac-CSHAVC-NH_2
HAVscr	Ac-HSVSAS-NH_2
ADT peptides	
ADT6	Ac-ADTPPV-NH_2
ADTC1	Cycloid(1,7)Ac-CADTPPVC-NH_2
ADTC5	Cyclo(1,7)Ac-CDTPPVC-NH_2

cadherin-cadherin interactions by cadherin peptides was first investigated to increase porosity of the BBB paracellular pathway in an equilibrium and reversible fashion. In this case, cadherin peptides derived from the homophilic contact regions of the protein that are responsible for the cadherin-cadherin interactions were designed as blood-brain barrier modulators (BBBMs). HAV and ADT peptides (Table 7.2) derived from the contact regions of the extracellular-1 (EC1) domain of E-cadherin have been shown to modulate the BBB intercellular junctions in vitro and in vivo.

Initially, cadherin peptides (HAV and ADT peptides; Table 7.1) were evaluated to modulate in vitro intercellular junctions of cell monolayers of bovine brain microvessel endothelial cell (BBMEC) (Lutz and Siahaan 1997a), MDCK (Makagiansar et al. 2001; Sinaga et al. 2002), and Caco-2 cells (Kiptoo et al. 2011; Calcagno et al. 2004). It was found that HAV and ADT peptides (Table 7.2) modulate the intercellular junctions of cell monolayers as indicated by lowering *trans*-epithelial electrical resistance (TEER) values of the MDCK and Caco-2 monolayers upon incubation with cadherin peptides. HAV and ADT peptides also enhanced the transport of ^{14}C-mannitol paracellular marker molecules across the cell monolayers from the apical side (AP) to basolateral side (BL) (Fig. 7.3a) (Makagiansar et al. 2001; Laksitorini et al. 2015). As an example, HAV6 peptide produced an eightfold enhancement in mannitol permeability compared to control, while the derivative



b

Peptide	$P_{app} \times 10^{-6}$ (cm/s)	Enhancement
Control	2.03 ± 1.5	1
Ac-SHAVSS-NH_2	17.0 ± 0.40	8
Ac-SHAVAS-NH_2	36.2 ± 0.20	17.8

Fig. 7.3 (**a**) Diagram of biological cell culture monolayers as an in vitro model of the BBB. ^{14}C-mannitol is used as a paracellular marker to evaluate the modulation of the BBB paracellular pathways by the BBBMs such as HAV peptides. (**b**) HAV peptides enhanced the permeation of ^{14}c-mannitol from the apical (ap) side to basolateral (bl) side of MDCK cells as an in vitro model of the biological barrier

HAV4 enhanced mannitol 17 times more than control in the MDCK cell culture model (Fig. 7.3b).

The first animal study carried out using HAV6 peptide (Table 7.2) to enhance brain delivery of ^{14}C-mannitol into the brain used the in situ rat brain perfusion method, which is a well-established method to study drug delivery across the BBB (Takasato et al. 1984; Neuwelt et al. 1984a). Infusions of ^{14}C-mannitol alone and ^{14}C-mannitol PLUS_SPI LABL6 peptide (EATDSG) were used as negative controls (Kiptoo et al. 2011). The data showed that HAV6 peptide significantly enhanced brain deposition of ^{14}C-mannitol compared to that of ^{14}C-mannitol alone or ^{14}C-mannitol PLUS_SPI LABL6 peptide.[14] This indicates that HAV6 modulates the BBB to increase the paracellular porosity that allows mannitol to pass through the BBB (Kiptoo et al. 2011).

To test whether HAV6 can enhance brain delivery of drugs that are Pgp substrates, the delivery of ^{3}H-daunomycin into the brain was evaluated using the in situ rat brain perfusion method (Kiptoo et al. 2011). Because ^{3}H-daunomycin is a substrate of Pgp, its infusion showed low deposition in the brain because it was

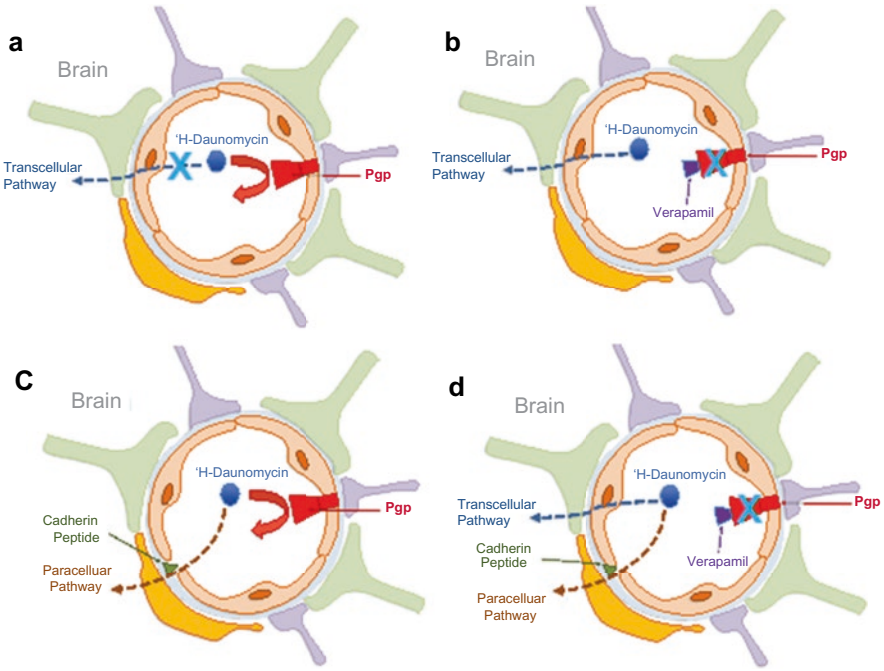


Fig. 7.4 Mechanism of ^{3}H-daunomycin transport across the BBB as Pgp substrate. (**a**) ^{3}H-daunomycin is a substrate of Pgp, and it was prevented by Pgp to diffuse through the BBB via transcellular pathway. (**b**) Inhibition of Pgp by verapamil enhanced transcellular diffusion ^{3}H-daunomycin across the BBB. (**c**) Modulation of the BBB intercellular junctions by cadherin peptides allows the penetration of ^{3}H-daunomycin through the BBB via the paracellular pathway. (**d**) Co-administration of ^{3}H-daunomycin with verapamil and cadherin peptide enhanced the BBB diffusion of ^{3}H-daunomycin via both trans- and paracellular pathways

prevented by Pgp to diffuse through the transcellular pathway (Fig. 7.4a). However, co-delivery of verapamil as a Pgp inhibitor with ^{3}H-daunomycin significantly enhanced passive diffusion of ^{3}H-daunomycin via transcellular pathway (Fig. 7.4b). A combination of ^{3}H-daunomycin and HAV6 significantly enhanced the brain deposition of ^{3}H-daunomycin compared to delivering ^{3}H-daunomycin alone. The results indicate that HAV6 improved the BBB penetration via paracellular pathway (Fig. 7.4c). Also, it was found that scramble HAV6 (HAV6scr; Table 7.2) did not improve the BBB permeation of ^{3}H-daunomycin, suggesting that the sequence specificity of HAV6 was necessary for BBBM activity (Kiptoo et al. 2011). A combination of HAV6 and verapamil led to higher delivery of ^{3}H-daunomycin compared to HAV6 or verapamil alone, suggesting improved BBB penetration of ^{3}H-daunomycin was via both transcellular and paracellular pathways (Fig. 7.4d).

Subsequently, HAV6 peptide has been shown to enhance brain delivery of camptothecin-glutamic acid (CPT-Glu) conjugate and gadopentetic acid (Gd-DTPA) as detected using LC-MS/MS and magnetic resonance imaging (MRI), respectively (Tabanor et al. 2016). A significantly higher amount of CPT-Glu was found in the rat brain after administration of CPT-Glu along with HAV6 via the rat's left carotid artery compared to CPT-Glu administration alone in in situ rat brain perfusion method (Tabanor et al. 2016). In the same study, Gd-DTPA, as an MRI enhancing agent, was administered via i.v. injections, and after 9 min, peptide or vehicle was administered via i.v. followed by monitoring the amount of Gd-DTPA in the brain by MRI (Tabanor et al. 2016). Immediately after the injection of HAV6 peptide, significantly higher depositions of Gd-DTPA in the rat brains were observed compared to those injected with vehicle (Tabanor et al. 2016). The significant increases were observed in the hippocampus, cerebellum, brain ventral, deep rostral, and deep caudal of the brain (Tabanor et al. 2016). These results support the idea that HAV6 peptide immediately increases the porosity of the BBB paracellular pathway to allow paracellular penetration of Gd-DTPA.

To evaluate whether BBBMs can also work to modulate the BBB in mice, rhodamine 800 (R800) and 25 kDa IRDye800CW-polyethylene glycol (IRdye800CW-PEG) were delivered via i.v. injections with and without HAV6 peptide (On et al. 2014). As in the daunomycin study, R800 was selected because it is a substrate for Pgp efflux pump (Fig. 7.4a), while IRdye800CW-PEG (25 kDa) was selected as a large molecule marker (On et al. 2014). Brain depositions of both molecules can be quantified using near IR fluorescence (NIRF) imaging. It was found that administration of R800 along with HAV6 significantly increased the brain deposition of R800 compared to administration of R800 alone. As in daunomycin, although R800 is a substrate for Pgp, HAV6 peptide increased the BBB permeation of R800 via the paracellular pathway (Fig. 7.4c). Administration of R800 along with Pgp inhibitor GF120918 enhanced the penetration of R800 via the transcellular pathway of the BBB compared to administration of R800 alone, confirming that R800 is a substrate of Pgp (Fig. 7.4b) (On et al. 2014). Furthermore, brain delivery of IRdye800CW-PEG was enhanced significantly by HAV6 peptide but not by GF120918 when compared to administration of IRdye800CW-PEG alone. GF120918 did not enhance the transport of IRdye800CW-PEG because IRdye800CW-PEG cannot cross through

the transcellular pathway due to its hydrophilicity. Overall, HAV6 can increase the delivery of efflux pump substrate R800 and a large molecule PEG via the paracellular pathway (On et al. 2014).

The activity of HAV6 peptide to deliver Gd-DTPA into the brain of Balb/c mice was studied in time-dependent manner by monitoring its brain deposition every 3 min for 42 min using MRI after i.v. administration (On et al. 2014). The results indicated that the brain depositions of Gd-DTPA were significantly higher (two–fourfold) when delivered with HAV6 compared to those administered with Gd-DTPA alone (On et al. 2014). In addition, enhancement of Gd-DTPA brain deposition was observed in the first 3 min after i.v. administration. The duration of the BBB pore opening generated by HAV6 was determined using a pretreatment experiment in which the mice were first treated with HAV6 peptide. After a certain time delay, marker molecules such as Gd-DTPA were administered. It is interesting to find that after 60 min delay, no enhancement of Gd-DTPA brain deposition was observed, indicating that the BBB modulation by HAV6 peptide was over in after 60 min. The results suggested that modulation of the BBB by HAV6 peptide is temporary and reversible.

Another BBBM called ADTC5 peptide (Table 7.2) was found to enhance brain delivery of ^{14}C-mannitol and Gd-DTPA in mice (Laksitorini et al. 2015). Similar to HAV6 peptide, the duration of BBB modulation by ADTC5 was also determined by pretreatment with ADTC5 followed by delivery of Gd-DTPA. Using Gd-DTPA, it was observed that the BBB paracellular pathway pore opening by ADTC5 was closed between 2 and 4 h, indicating longer paracellular opening compared to HAV6 peptide. ADTC5 peptide can also enhance the brain deposition of cIBR7 peptide (Cyclo(1,8)CPRGGSVC) in rats and IRdye-800cw-labeled cLABL peptide (IRdye-800cw-Cyclo(1,12)PenITDGEATDSGC) in mice as quantitatively determined by mass spectrometry and NIRF imaging, respectively (Ulapane et al. 2017).

It is interesting to find that ADTC5 peptide enhanced brain delivery of various size proteins, including 15 kDa lysozyme, 65 kDa albumin, and 150 kDa IgG monoclonal antibody (mAb) (Ulapane et al. 2019b). However, ADTC5 cannot enhance the delivery of 220 kDa fibronectin, suggesting that there is a size limit of protein that can be delivered to the brain by ADTC5. The data showed that ADTC5 peptide was better than HAV6 peptide in delivering lysozyme and albumin across the BBB (Ulapane et al. 2019b). The duration of BBB opening depended on the size of the delivered molecule. For example, during BBB pretreatment using BBBM peptide before delivering the marker molecule, it showed that the paracellular pathway pore opening by ADTC5 lasted about 40 min when delivering a small molecule such as Gd-DTPA. However, the BBB paracellular opening was less than 10 min for the larger 65 kDa albumin permeability marker. It hypothesized that the BBBMs disrupt the intercellular junctions to generate large, medium, and small pores. The large pores collapse rapidly to medium followed by the conversion of medium pores to small pores in time-dependent fashion. Thus, the BBB paracellular opening for large- and medium-size molecules is shorter than for small molecules (Ulapane et al. 2017).

7.8 Nasal Delivery of Peptides

An intranasal delivery method is one way to enhance delivery of peptides into the brain by bypassing the BBB in a noninvasive manner. Some peptides and proteins such as oxytocin and insulin have delivered intranasally to reach phase IV clinical trials for neurodegenerative diseases such as Alzheimer's disease and related brain diseases (Samaridou and Alonso 2018). Thus, many peptides and proteins have been extensively investigated for their brain delivery via nasal administration to potentially treat neurodegenerative diseases (Meredith et al. 2015). One of the advantages of nasal delivery method is that it avoids peptide degradation in the blood as well as it can target a specific brain region such as an olfactory bulb and its surrounding brain regions (Meredith et al. 2015). Thus, nasal delivery could be more favorable than a systemic delivery via i.v. administration because during i.v. administration the peptide could be degraded and cleared in the systemic circulation.

To improve nasal delivery of peptides, many strategies have been developed. In general, peptides had to overcome nasal mucosa, olfactory epithelium barrier, and the cribriform plate before entering the olfactory bulb of the brain (Fig. 7.5) (Meredith et al. 2015; Samaridou and Alonso 2018). The peptides also had to survive enzymatic degradation at the olfactory epithelium. There are three ways that peptides can enter the brain. First, peptides enter the nasal cavity followed by crossing transcellular or paracellular pathway of the olfactory epithelium as well as crossing the cribriform plate into the olfactory bulb. Second, peptides enter the nasal cavity and are absorbed into the blood vessels of the systemic circulation followed by crossing the BBB into the brain. Third, the peptide could diffuse through

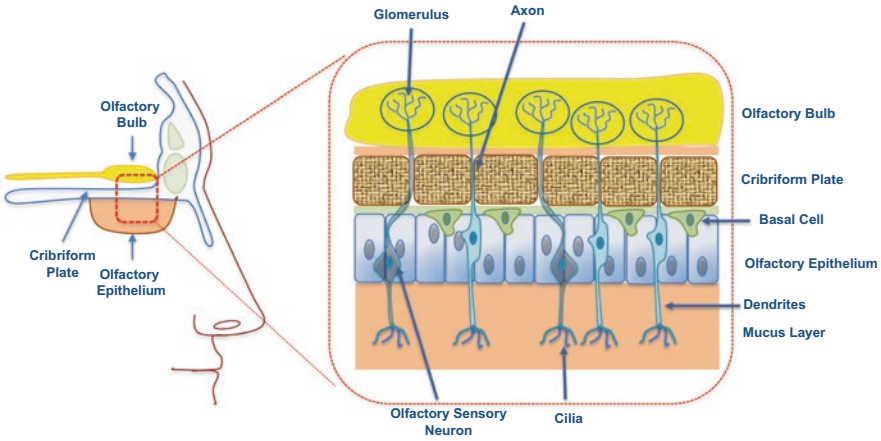


Fig. 7.5 The structure of nasal cavity as route for the delivery of peptides via intranasal delivery. The peptides need to cross the olfactory epithelium and cribriform plate to enter the olfactory bulb of the brain. The structure of the epithelium is decorated with sensory neurons with dendrites and cilia along with axons penetrating cribriform plates into the olfactory bulb

neuronal axons of the trigeminal nerve (Fig. 7.5) (Samaridou and Alonso 2018). Although the trigeminal nerve is one of the proposed transport pathways for peptides into the brain, this pathway has not been fully investigated. It has been proposed that the paracellular pathway is the major route for peptides to cross the epithelial olfactory barrier and through the cribriform plate (Fig. 7.5). It has been shown that tight junction disruptors such as carnitines and ultrasound improved peptide intranasal delivery. Cell-penetrating peptides (CPPs), receptor-mediated endocytosis process, and nanoparticles have also been explored to enhance transcellular transport of peptides through the nasal olfactory epithelium.

Oxytocin has been delivered intranasally for treating autism spectrum disorder (ASD) in clinical trials. The pharmacokinetics and brain distribution of oxytocin have been fully investigated (Tanaka et al. 2018). The nasally administered oxytocin can effectively reach the brain compared to those administered intravenously and intraperitoneally (Tanaka et al. 2018). The majority of oxytocin was found in the olfactory bulb followed by the frontal and occipital halves of the brain. Because the site of action for treatment of ASD is near the olfactory bulb, the intranasal delivery is suitable for this purpose (Tanaka et al. 2018). During nasal delivery, oxytocin was chemically stable and can be absorbed across the nasal epithelium (Tanaka et al. 2018). The plasma concentrations of the intranasally delivered oxytocin were much lower than intravenous (Tanaka et al. 2018). In a stress mouse model that is similar to ASD, intranasal delivery of oxytocin lowered the plasma levels of a stress hormone corticosteroid compared to that of i.v. administered oxytocin, implying the nasal delivery was more effective than that of i.v. administration (Tanaka et al. 2018).

Vasoactive intestinal peptide (VIP) has been formulated for nasal delivery with optimum stability in 1% bovine serum albumin concentration (BSA) and 0.1% lauroylcarnitine (LC) (Dufes et al. 2003). Nasal delivery of VIP was also more effective than i.v. administration with high deposition in the cerebellum, central gray, amygdaloid nuclei, and thalamic, hypothalamic, and olfactory bulbs where VIP receptors are located (Dufes et al. 2003). The nasal delivery of VIP was pH dependent, and the best brain uptake of ^{125}I-VIP was at pH 9 as detected by radioactivity levels in the brain (Dufes et al. 2003). The presence of LC in the formulation increased brain uptake of VIP at pH 4 and 7; this was due to the effect of LC in disrupting the BBB tight junctions by lowering the expression levels of tight junction proteins such as claudins (e.g., claudin 1, 4, and 5) (Doi et al. 2011). During i.v administration, a low amount of intact VIP was found in the brain, and most radioactivity found distributed throughout the whole brain emanated from ^{125}I-VIP degradation products because VIP was degraded rapidly in the blood (Dufes et al. 2003). In contrast, intact ^{125}I-VIP was found in the brain when delivered via nasal route. These results suggest that nasal peptide delivery was better than i.v. delivery and the penetration of VIP was via the paracellular pathways of the olfactory epithelium.

Pituitary adenylate cyclase-activating polypeptide (PCAP) alone can cross the BBB when administered via i.v. route; however, the transport across the BBB was not effective due to its recognition by an efflux pump and degradation in the blood. Alternatively, PACP in formulation with six monosaccharide cyclodextrin delivered

intranasally enhanced brain deposition at the olfactory bulb but not the other brain regions (Meredith et al. 2015). On the other hand, cyclodextrin with seven monosaccharides increased PCAP levels in the whole brain except the olfactory bulb and striatum (Meredith et al. 2015). Although the mechanism of action of these cyclodextrins is still not clear, it has been proposed that they interact with cholesterol of cell membranes to disrupt cell membrane integrity of the olfactory epithelium to improve peptide permeation through para- and transcellular pathways (Kiss et al. 2010; Hussain et al. 2003). Similarly, intranasal co-administration of galanin-like peptide (GALP) with cyclodextrin resulted in its deposition on all brain regions, while administration of GALP alone resulted in a high deposition in the olfactory bulb only.

Other peptides such as exendin, insulin, and leptin-like peptide have been found to enter the brain when administered via intranasal route with higher brain deposition in the olfactory bulb compared to those delivered via i.v. administrations. In general, intranasal delivery increased the peptide deposition at the olfactory bulb better than other brain regions. As with other biological barriers, peptides and proteins have physicochemical properties that are not conducive to cross via the transcellular pathway of the olfactory epithelium; thus, the transport pathway through epithelium is most likely via the paracellular route. For effective brain delivery, the peptide should penetrate the mucosa epithelium and withstand enzymatic degradation in the mucosa epithelium. Because of the limited delivery space of the olfactory epithelium, peptides had to be delivered in a high concentration with less than 400 μL of delivery volume using intranasal liquid delivery systems. The role of permeation enhancers such as cyclodextrins, CPP, and others became important for intranasal peptide brain delivery.

Nanotechnology has also been utilized for intranasal delivery of peptides to the brain because nanoparticles can encapsulate a high dose of peptide as well as provide peptide protection from enzymatic degradation in the mucosa epithelium. Nanoparticles have been constructed from various materials, including polylactic/glycolic acid, chitosan, gelatin, or cationic liposomes. Liposomes, with size around 100 nm, have extensively been used to deliver peptides intranasally to olfactory bulb through the axons (Samaridou and Alonso 2018). Because mucus has negative charges, it is preferable that the nanoparticles have positive charges for their diffusion through nasal mucus layers and olfactory epithelium (Fig. 7.5). The presence of surfactants (e.g., Tween 80, polysorbate 80, poloxamer 188) can improve the penetration of peptide- or protein-loaded nanoparticles; they presumably behave as membrane disruptors that allow nanoparticle penetration through paracellular pathways of nasal epithelium. Pegylated liposomes have been developed to deliver H102 peptide for treatment of Alzheimer's disease in a rat model. In addition, substance P peptide was successfully delivered to the brain using gelatin-lipid nanoparticles via nasal delivery route. In summary, the physicochemical properties of the nanoparticles (i.e., size, charge, composition of surface) influence their penetration across the nasal epithelium into the olfactory bulb.

For receptor-mediated transport, the surface of nanoparticles was decorated with peptides or proteins for improving selectivity and uptake by nasal epithelium.

Lactoferrin has been used to decorate nanoparticle surfaces for nasal delivery of neuropeptide (Neuwelt et al. 1985). The function of lactoferrin is to target lactoferrin receptors, which are highly expressed in the brain (Samaridou and Alonso 2018). CPP-decorated chitosan nanoparticles have also been used to improve brain delivery of neurotrophic factors via intranasal administration.

7.9 Conclusions

Delivery of drug and diagnostic molecules to the brain is still challenging; however, many advancements have been made to deliver small up to large molecules across the BBB and into the brain. Osmotic BBB disruption method has been successfully used to deliver anticancer drugs to brain tumor patients. Preclinically, BBBM peptides have been used to deliver various proteins to the brain in animal models of MS, Alzheimer's disease, and brain tumors; however, more work is needed to evaluate the side effects of this method. Intranasal delivery of peptides to the brain has shown some success in the preclinical and clinical studies with the hope that the method can be used to effectively deliver various peptides to the brain. Thus, there is a hope that some of these methods can help advance the development of therapeutic and diagnostic agents for brain disease.

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