A REVIEW OF CELL-PENETRATING PEPTIDES MEDIATED DRUG DELIVERY TO THE BRAIN

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A thesis submitted to the School of Pharmacy in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy

School of Pharmacy
Brac University
February, 2023

Declaration

It is hereby declared that

1. The thesis submitted is my/our own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

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Approval

The thesis titled "A review of Cell-penetrating peptide mediated drug delivery to central nervous system (CNS)" submitted by Zarin Tasnim Tisha (19146088), of Spring'2019 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy.

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Ethics Statement

This study does not involve any human and animal trial.

Abstract

The central nervous system (CNS) is prone to different CNS disorders such as stroke, tumor, different neurodegenerative diseases such as Parkinson's, Alzheimer's disease, Huntington's diseases etc. There are several treatment strategies available such as surgery, radiotherapy, chemotherapy, chemotherapeutic drugs etc. But the physiological barrier of CNS, the blood-brain barrier (BBB) poses as a main hindrance in these treatments. In this regard, cell-penetrating peptides (CPPs) mediated approach can be a promising treatment strategy to deliver the drug to brain crossing the BBB. CPPs are small peptides containing positive charge, can cross the cellular membrane and translocate different cargos containing protein, peptides, nucleic acids. They also have proven their ability to cross the BBB and deliver the cargos for efficient neural drug administration. They have less side effects and cytotoxcity. In this review, the main objective is to explore the potentiality of CPPs in delivering the drug to CNS for CNS disorders.

Keywords: Cell-penetrating peptides, central nervous system, neurodegenerative diseases, blood-brain barrier, cytotoxicity

Dedication

Dedicated to my parents

Acknowledgement

First and foremost, I would like to express my gratitude to the Almighty for his endless gifts, which have been given to me in an effort to provide me with the strength and determination to complete this project.

It is my genuine pleasure to offer my heartfelt appreciation to my academic supervisor, Sabrina Sharmin (Assistant Professor at Brac University's School of Pharmacy), for her invaluable guidance and encouragement during this research. Through the course of my education and project writing, she was a true source of advice and support for me. I am quite grateful to her for her valuable comments and ideas during my study, which helped me much in completing my project work in a timely manner.

Also, I would like to express my gratitude for the support, inspiration, and leadership of

Professor Dr. Eva Rahman Kabir (Dean, School of Pharmacy, Brac University) and Professor Dr. Hasina Yasmin (Program Director and Assistant Dean, School of Pharmacy, Brac University).

Finally, I'd like to convey my thanks to my parents, who never cease to inspire me to push myself beyond my comfort zone. I would not have made it this far without the daily prayers and unconditional love of my family and loved ones. I'd also want to express my gratitude to all of the folks who, whenever they were called upon, went above and beyond to assist me.

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List of Acronyms

AA	Amino acids
AAV	Adeno-associated virus
ALS	Amyotrophic lateral sclerosis
AMPs	Antimicrobial peptides
BBB	Blood-brain barrier
CNS	Central nervous system
CPPs	Cell-penetrating peptides
ECs	Endothelial cells
GFP	Green fluorescent protein
GAGs	Glycosaminoglycans
HS	Heparan sulfate
HA	Human astrocytes
HIV-1	Human immunodeficiency virus type 1
IBD	Inflammatory bowel movement disorders
JAMs	Junctional adhesion molecules
LMWP	Low molecular weight protamine
MTX	Methotrexate
MMPs	Matrix metalloproteinases

NF	Nuclear factor
NEMO	NF-κB essential modulator
NBD	NEMO binding domain
NVU	Neurovascular unit
NC	Negative control
ND	Neurodegenerative disorders
NA	Nucleic acids
NMR	Nuclear magnetic resolution
NPs	Nanoparticles
PTD	Protein transduction domain
siRNA	Small Interfering Ribonucleic acid
TAT	Trans-activator protein

Chapter 1

Introduction

1.1 Background of Cell-penetrating peptides

The nature of peptides and proteins are hydrophilic in nature which limits their permeability in cell membranes. To successfully deliver these, Cell-penetrating peptides are promising tool and have been successfully uses for protein delivery across the different epithelial cells as well as blood-brain barrier(BBB) (Kristensen et al., 2016).In 1988, discovery of purified trans-activator protein (TAT), from human immunodeficiency virus type 1 (HIV-1), first CPPs was discovered by Frankel and Pabo (Ramsey & Flynn, 2015). According to research, the majority of CPPs are less than 40 amino acids and are readily absorbed by cells without generating cell toxicity. CPPs were once believed to be comparable to membrane-interacting antimicrobial peptides (AMPs) based on their structural and functional similarities. In the 1990s, two CPPs were discovered: penetratin and trans-activating factor TAT from human immunodeficiency virus type 1. Because of their short size, amphipathicity, widely scattered cationic charges, and most critically its secondary structure- all facilitate the initial peptide-membrane contact and subsequent cellular absorption, so making CPP a cargo delivery agent (Boisguérin et al., 2015)(Bera & Bhunia, 2019). In 2000, pre-clinical studies of CPPs started with the aim of understanding about cellular penetration and their pharmacokinetic properties. First chimeric fusion was combination of Antp and a fragment of caveolin 1 peptide. This was developed to see anti-inflammatory properties of CPPs in mice. Then, a series of studies were performed to explore the performance of CPPs in various tissues. For example, conjugation of doxorubicin with two CPPs, D-Penetratin and SynB1 showed that the cargo reaches to parenchyma of brain by crossing BBB and increases the drug concentration compared to unmodified version of drug(Kurrikoff et al., 2016).CPPs have

been proven to have a variety of both therapeutic and biological applications, including cancer and enzyme replacement treatment, vaccine development, inflammation, diagnostics, and even nose-to-brain transmission for central nervous system disorders (Hasannejad-Asl et al., 2022). The standard constituents of a CPP are a CARGO peptide and an effector peptide. As seen in Figure 1, the first peptide increases intracellular penetration, whereas the second peptide inhibits a key protein-protein interaction and initiates a physiological response (Antoniou & Borsello, 2010). Translocation of macromolecules without disrupting the cellular membrane is one of the most unique traits of CPPs and this trait makes it less toxic and highly desired for drug delivery (Foged & Nielsen, 2008).

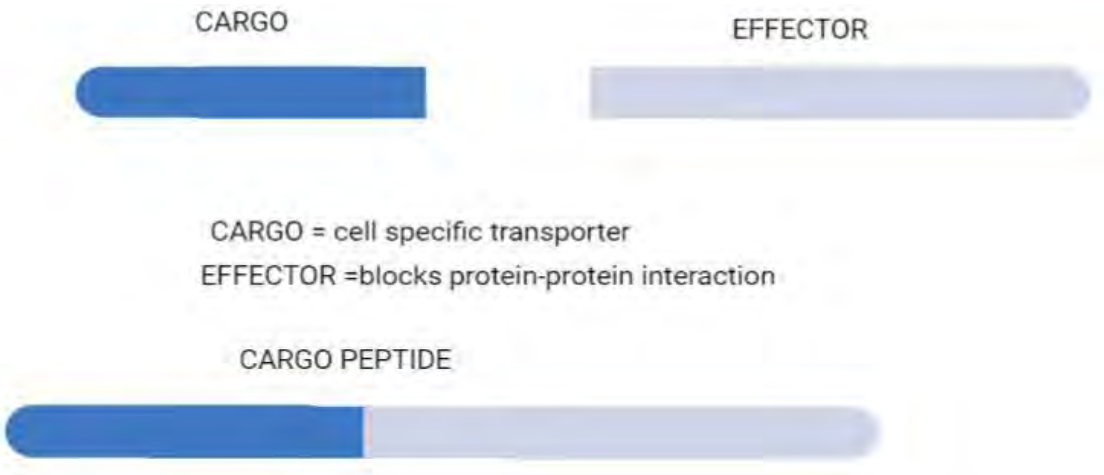

Figure 1: A typical Cell-penetrating peptide with a Cargo molecule (blue) and an effector (light blue) (Antoniou & Borsello, 2010).

1.2 Aim of the project

Brain and nerve system illnesses are among the most disabling, with effects that may last a lifetime. Modern biomedical technology has made it possible for more effective medical treatments, yet the progression of noninvasive therapeutic alternatives remain limited due to the unique biological environment and complicated structure of the CNS. Therefore, it is crucial to develop a carrier that enhances the delivery of therapeutic medications into the brain without disrupting the BBB. And, CPPs can be considered as one of the candidates. The aim of this literature review is to examine the potentiality of cell-penetrating peptides in the treatment of central nervous system disorders.

1.3 Objectives of this study:

The objectives of this study are:

- To learn about how CPPs work
- To give an insight about how CPPs can be a potential candidates for drug delivery to CNS for CNS-based disorder
- To explore more about future aspects and challenges of CPPs-mediated drug delivery

Chapter 2

Methodology

The resources were collected from published peer-reviews studies, research articles found in different well-known databases such as PubMed, ScienceDirect, Nature, Google Scholar, MDPI, Cell, Frontiers etc. The articles were searched based on important keywords for example, “Cell-penetrating peptides”, “Central nervous system” etc. Relevant articles were collected and background information was reviewed based on this topic. After that, a research gap was identified by going through all the articles. After deciding the topic, an outline was prepared with relevant headings and subheadings. During writing process, information was properly paraphrased and cited. The in-text citation and the bibliography were generated by using Mendeley Desktop.

Chapter 3

Overview of Central nervous system (CNS) and CNS diseases

3.1 Anatomy of Blood-brain barrier (BBB):

The brain, which is the most critical and sensitive organ in our body. It is highly regulated to maintain the homeostasis within the CNS for proper functioning of brain. To maintain the homeostasis, the neural environment requires a tight regulation of cells, ions and molecular transportation between the brain and blood (Serlin et al., 2015). This tight regulation is highly maintained by a unique physical barrier known as Blood-brain barrier (BBB) and the Blood cerebrospinal fluid (BCSF) (S. Zhang et al., 2022). BBB is mainly composed of highly specialized endothelilal cells, basal membrane, pericytes and astrocyte endfoot. There is a tight junction (Figure 2) among the endothelial cells which restricts the entrances of different substances in the brain. The tight junction is composed of three types of transmembrane proteins such as JAMs (Junctional adhesion molecules), caludin, occludin and cadherins which works as adhesion junction. This four proteins are important for paracellular permeabilities (Gupta et al., 2018)(Wilhelm et al., 2013).The basic and structural unit of the CNS is neurovascular unit (NVU) which is composed of cells of BBB, capillary and an astrocyte that helps in communication between the neuron and its surrounded capillary. The NVU maintains the transfer of materials from blood to CNS as well as sends the waste material back to vasculature. BBB has large surface area with a weight of 1.3-1.4 kg. The volume of human cortex is $1mm^3$ and this contains 10 cm^2 surface area of microcirculation.

This huge surface area of the BBB is an important structure for normal functioning of the CNS and can be used as a potential target area of drug delivery (Yuan et al., 2020).

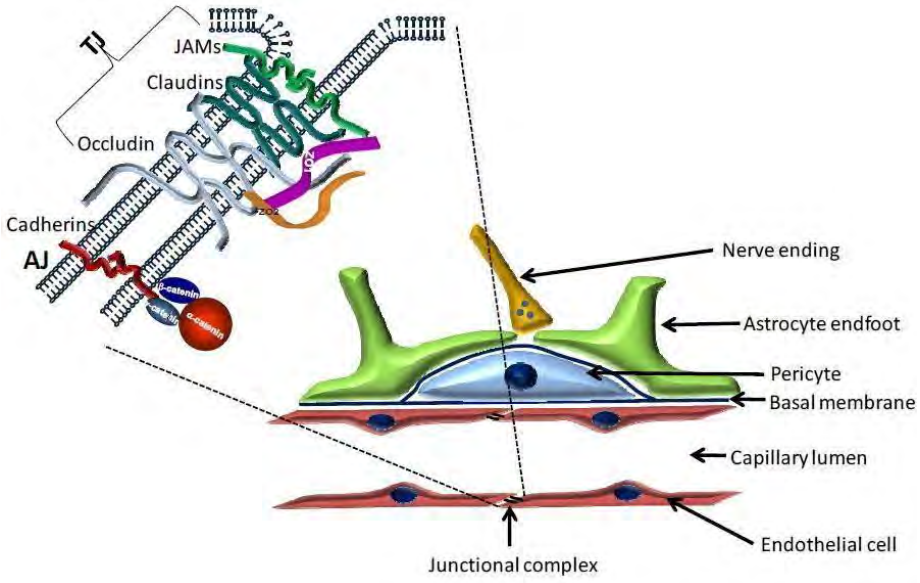

Figure 2: Schematic representation of tight junction (TJ) of BBB (Wilhelm et al., 2013).

3.2 Some common CNS diseases

Neurological illness is a major concern generally observed in older patients. Stroke, dementia as well as epilepsy- are the leading causes of death and disability among the working-age population (Birbeck et al., 2015). Epilepsy causes seizures and the cause of this disease is still unclear which makes it difficult to treat (Riva et al., 2021). Neurodegenerative disorders (NDs) are comprised of different group of diseases that cause partial or full loss of the functions of neurons (Morén et al., 2022). Neurodegenerative disorders like Alzheimer's and Parkinson's diseases, represent a grave threat to human health. Age-related disorders such as dementia and Alzheimer's are on the rise, in part owing to the increase in the elderly population in recent decades. Alzheimer's, Parkinson's, Huntington's, amyotrophic lateral sclerosis (ALS), frontotemporal dementia, and spinocerebellar ataxias, to mention a few, are

neurodegenerative diseases. Some of them induce mental deterioration, while others make it difficult to move, speak, or breathe (Mortada et al., 2021) (Lamptey et al., 2022). Because of dementia, there is a decline of our cognitive ability interfering our daily activities. Alzheimer's disease is the most common type of dementia (Kumar Thakur et al., 2018). Parkinson's disease is the common ND which can be caused because of genetics, environmental factors (Davie, 2008). However, the extraordinarily effective blood-brain barrier (BBB) remains a significant barrier to the effective treatment of several CNS illnesses (Chen et al., 2020) (Lamptey et al., 2022).

Chapter 4

Overview of Cell-penetrating peptides (CPPs)

One of the major challenges of delivering large molecules inside the cells is their low permeability. CPPs have been one of the breakthrough discoveries for last 20 years because of its capability of internalizing molecules into the cells without being toxic (Jobin et al., 2015).

4.1 Classifications of cell-penetrating peptides

4.1.1 Classification based on the source of peptides

The peptide's origin is a relevant classification criterion for CPPs. Firstly, some peptides are generated from natural proteins, also known as protein transduction domains (PTDs). Examples of these peptides include TAT and penetrating. DNA-RNA-binding proteins, viral particle envelope proteins, antimicrobial proteins, gene transcription transactivators, and plant circular skeletal proteins are examples of natural CPPs (Kardani et al., 2019). Secondly, the chimeric peptides that have two or more motifs from separate peptides fused together. Transportan, which is composed of mastoparan and galanin, and its shorter counterpart

TP10are examples of chimeric peptides. And finally, 3) manufactured peptides that inclued polyarginine family (Graslund et al., 2011) (Kardani et al., 2019). Synthetic or manufactured CPPs that mimic the structure of natural CPPs (Holm & Langel, 2005); examples include polyarginine and modeled amphipathic peptide (MAP) (Kardani et al., 2019).

4.1.1 Classification based on physicochemical properties

CPPs contain a wide range of sequences with minimal homology or overlap, yet they nevertheless fall into two main categories: tissue-specific and non-tissue-specific. Cationic CPPs, hydrophobic CPPs, and amphipathic CPPs are subsets of the non-tissue specific CPP category. Tat and penetratin, the first two CPPs discovered, are both examples of cationic peptides (Frøslev et al., 2022). Homo-polymers of Arginine and Lysine, as well as the DNA-binding protein from herpes simplex virus type 1, VP22, are further examples of cationic peptides (Table 1). Research on Arginine-based homo-polymers (of lengths ranging from R3 to R12) has demonstrated that a sequence of six arginines is sufficient for cellular absorption, and that an increase in the number of Arginine residues improves transduction effectiveness. While it has been previously found equivalent cellular absorption has been observed with both 8-mer homo-polymers of lysine or arginine, poly-lysine revealed decreased uptake in the current investigation. There is a decline in transduction efficiency for Arginine and Lysine homo-polymers greater than 12 amino acids (Zahid & Robbins, 2015) (Kardani et al., 2019). The positive charge of cationic CPPs demonstrates high affinity for the cytoplasmic membrane under normal physiological pH values. Through electrostatic contact, cationic CPPs bind to the negatively charged cell membrane glycoprotein, and the resulting complex is internalized into the cell without the involvement of a receptor (Xie et al., 2020).

More than 40% of all CPPs discovered to date are amphipathic CPPs. Both polar and non-polar portions of amino acids are present in amphiphilic CPPs, with the latter being particularly prevalent in hydrophobic amino acids (for example, alanine, valine, leucine, and isoleucine). To facilitate their translocation through the cell membrane, amphipathic CPPs have both lipophilic and hydrophilic domains (Xie et al., 2020). Many amphipathic CPPS were created by covalently attaching a hydrophobic domain to a nuclear localization signal (NLS). Two examples of such peptides are MPG (GLAFLGFLGAAGSTMGAWSQPKKKRKV) and Pep-1 (KETWWETWWTEWSQPKKKRVK), both of which are derived from the nuclear localizing signal PKKRKV of SV40. Other main amphipathic CPPs including pVEC, ARF (1-22), and BPrPr (1-28) are all generated from natural proteins (Zahid & Robbins, 2015). (Kardani et al., 2019).

There aren't too many CPPs that are hydrophobic, and the ones that are tend to include a lot of uncharged amino acids or a small number of charged ones (less than 20% of the sequence) (Xie et al., 2020). Signal peptide sequences or vast peptide libraries shown on phage, plasmid, microorganism surfaces, or ribosomes are mined for hydrophobic CPPs (Zahid & Robbins, 2015). C105Y, Bip4, and K-FG are examples of naturally occurring hydrophobic CPPs (Xie et al., 2020).

Table 1: Different types of CPPs (Zahid & Robbins, 2015) (Xie et al., 2020).

Category	Peptides	Sequence	Origin
Cationic CPPs	TAT	RKKRRQRRR	Protein derived
	DPV3	RKKRRRESKKRRRES	
	DPV6	GRPRESGKKRKRKRLKP	
	Penetratin	RQIKIWFQNRRMKWKK	
	8-Arginine	RRRRRRRR	Synthetic
	8-Lysine	KKKKKKKK	n/a
Amphiphatic CPPs	pVEC	LLIILRRRIRKQAHAHSK	Protein derived
	ARF(19-31)	RVRVFWHIPRLT	
	Transportan	GWTLNSAGYLLGKINLKALAALAKKIL	
	MAP	KLALKLALKALKAALKLA	Synthetic
Hydrophobic CPPs	Bip4	VSALK	Protein derived
	C105Y	CSIPPEVKFNPFVYLI	

4.2 Cellular uptake of CPPs:

There are two types of formulation approaches of CPPs: Covalent conjugation and physical complexation. In covalent conjugation, CPPs is conjugated with protein cargos by disulfide or amine bonds or linkers. The linkers help to release the cargo after internalization. In covalent conjugation, CPP with its cargo represent well-defined molecular structure thus facilitating further researches. On the other hand, hydrophobic or electrostatic interaction between the CPP and cargo is known as physical complexation. Besides, non-covalent interaction depends on the physic-chemical properties of both the CPP and the cargo as well as the process of formulation (Kristensen et al., 2016). Though cellular uptake mechanism of CPPs has not been fully understood, two mechanisms are widely known, direct penetration

and endocytosis (Koren & Torchilin, 2012). Following the endocytosis, endosomal escape is really important otherwise degradation takes place. So, before degradation, cargo must escape from the endosome. This mechanism is also not fully understood. There are some suggested mechanisms by which endosomal escape happens. One of them is proton sponge effect in where endosome swells up and ruptures resulting releasing the cargo (Koren & Torchilin, 2012).

4.3 CPPs and Nanoparticles (NPs):

Combination of CPPs and nanoparticles (NPs) is valuable strategy (Gessner & Neundorf, 2020). Silver nanoparticles (AgNPs) have been widely applied in cancer treatment due to better stability, biocompatibility and strong anti-microbial properties but with increased size, cytotoxcity increase. Besides, gold NPs is also being explored to overcome the limitations of AgNPs(Arib et al., 2022)(Tabujew et al., 2015). One of the safest strategies to deliver AgNPs into the cells is using CPPs (Mussa Farkhani et al., 2017). Besides, to overcome the limitation of the oral delivery of peptide-based drugs, cyclic R9-CPP, a polyarginine rich CPPs with polylactide acid nanoparticles have been used and showed increased bioavailability (Uhl et al., 2020). The CPPs and nanoparticle combination can be formed by non-covalent or covalent bond (Figure 3). For carboxyl, sulfhydryl, amino etc. reactive groups prefers covalent bonding through thioester or disulfide bond. Linkage generally reduces the distance between CPPs and nanoparticles. Covalent bond may alter biological activity of the conjugation. On the other hand, non-covalent forms complex between them when they are mixed (Y. Zhang et al., 2021).

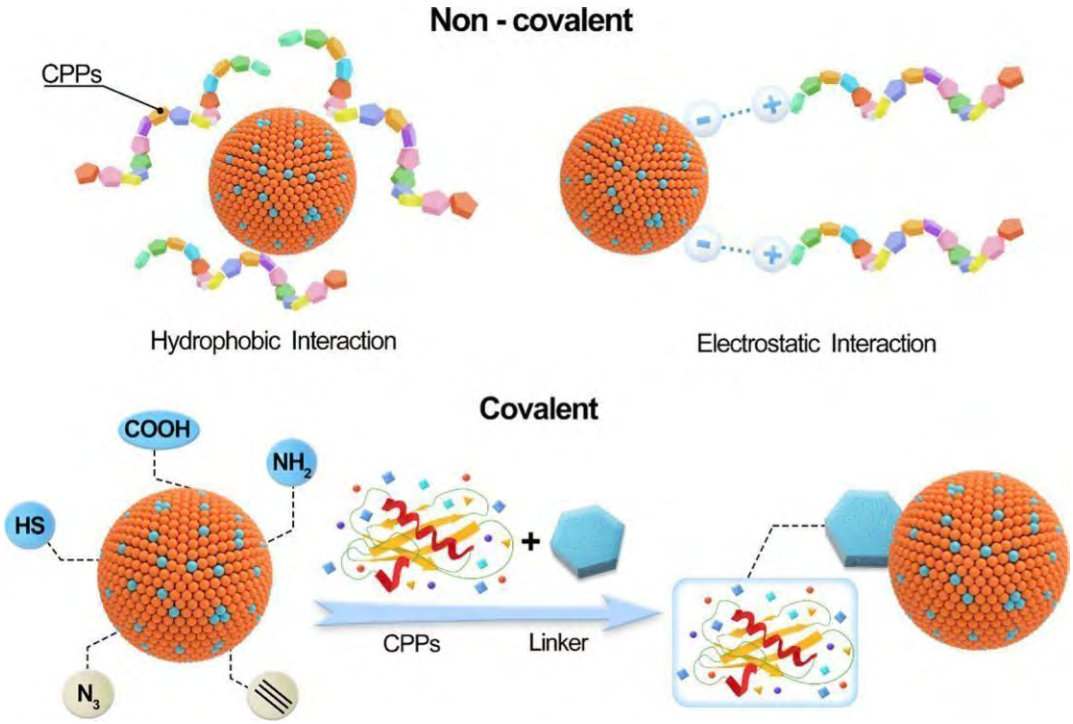

Figure 3: Conjugation between CPPs and nanomaterials (Y. Zhang et al., 2021).

Though nanoparticles have been researched for many years, NPs alone can increase drug accumulation in the target organs. To reduce this side effect, conjugation theory was proposed. As CPPs does not disturb biological barriers, thus CPPs with NPs can easily overcome the biological barriers. In table 2, conjugated CPPs with NPs are discussed (Kebebe et al., 2018) (Liu et al., 2022).

Table 2: Different CPPs-NPs conjugate and their functions (Liu et al., 2022).

Barrier	Conjugation of CPP-NPs	Sequence	Functions
Ocular barrier	POD-PEG NP	GGGG(ARKKAAKA)	Enhancing the outer nuclear layer thickness
Mucous barrier	Penetratin with mesostructured silica NP	RQIKIWFQNRRMKWKK	Promoting mucous permeability
BBB	R8-liposome; R8/Tf-liposome	RRRRRRRR	Increasing drug delivery to BBB
Skin barrier	MEL/TAT-MEL/PEO-b-PCL gel-like nanovehicle	YGRKKRRQRRR	Enhancing transdermal delivery

4.4 CPPs based therapeutic

4.4.1 CPPs in Cancer treatment:

CPPs are capable of delivering chemotherapeutic agents, nucleic acids and proteins as anti-cancer treatment. In cancer, multiple drug resistance (MDR) can be overcome by CPPs. It has been found that taxol bound to R8 CPP by disulfide bond, increases the the pharmacokinetics of the drug as well as overcomes the MDR compared to drug when used alone. Besides, methotrexate (MTX), another chemotherapeutic drug combined with YTA2 and YTA4 CPPs has shown improved performance in killing cancer cells than MTX alone (Borrelli et

al., 2018). Moreover, gene therapy can also benefit from CPPs as gene therapy is a good candidate for cancer treatment in future. The main limitation of gene therapy is the nucleic acids are large and hydrophilic thus they cannot cross the plasma membrane. So, they need a vector which will help them to cross the cell membrane. The cationic CPPs like TAT can bind to nucleic acids by a covalent or non-covalent bond, thus overcoming the limitations. Another emerging anti-cancer treatment strategy is protein delivery. TAT is well established to deliver several large protein molecules (120kDa β-galactosidase) into several organs. It has been found that Penetratin bound to cytotoxic T lymphocyte epitopes that derived from ovalbumin increases the stimulation of CD4+ and CD8+ T cells resulting inhibition of melanoma cell growth both in vivo and in vitro (Habault & Poyet, 2019). Besides, peptides are being used as imaging probes for diagnostic purposes because of their uptake specificity, increased affinity of binding, stability and fast clearance from non-specific targets. Protease is an enzyme which is seen increased amount in cancer cells. CPPs conjugated with optical imaging moieties for example, activable probes, fluorohore can be used as diagnostic tools in cancer treatment. Activable probes have flurophrobes which can cleave due to the presence of protease enzyme and this cleavage causes fluorescence intensity in cancer cells.Thus CPPs shows an immense possibility as a diagnostic approach in cancer treatment (Tripathi et al., 2018). KAFAK is an anti-inflammatory CPPs (Bartlett et al., 2013).

4.1.1 CPPs in inflammation:

Cosmeceutical peptides are used as anti-aging, anti-inflammatroty purposes. But as peptides are larger molecules, they cannot easily penetrate the skin. Here, CPPs have showed promising performance in artificial human skin to deliver the peptide cargos (Fu et al., 2020). Nuclear factor-κB (NF-κB) plays a major role in inflammatory responses. Interleukin-1 (IL-

1) and tumor necrosis factor (TNF) activate NF-κB and activates inflammatory responses for rheumatoid arthritis, inflammatory bowel movement disorders (IBD) etc. (Guo et al., 2016). Most of the inflammatory treatment is based on blocking the activation of NF-κB (Wang et al., 2011). NEMO binding domain (NBD) conjugated with CPP has shown downregulation of inflammatory factors in IBD mouse model. So, it has being considered as a treatment strategy of IBB (Guo et al., 2016).

4.4.2 CPPs for Vaccination:

Vaccination has been used as a preventative treatment strategy against infectious diseases. In live attenuated vaccine, weak pathogen is used to induce immune response and needs multiple doses for standard efficacy. Then, vaccines containing whole pathogen trigger unwanted side effects. Because of these limitations, recent focus is based on subunit vaccine development approach. In this case, CPPs can work as a potential delivery system as they have high cellular permeability without any receptors (Yang et al., 2019). In subunit and nucleic acids (NAs) vaccines, only specific antigens are used to formulate the vaccines resulting better safety profile than conventional ones (Hasannejad-Asl et al., 2022). Besides, cancer vaccination based on peptides is being widely researched and has shown modest success rate. To enhance the potency, peptides conjugated with CPPs are being explored for enhanced intracellular vaccine delivery (Backlund et al., 2022). In addition to, there are still no vaccines against viral infections which are major threat for human health. Nowadays, protein based vaccination against virus infection has showed its potentiality. Since antiviral agents and proteins have weak cellular permeability, CPPs delivery system can be used for successful delivery (Delcroix & Riley, 2010) (Sadeghian et al., 2022).

Chapter 05

CPPs mediated drug delivery to CNS

5.1 Barrier of drug delivery to CNS:

CNS has complex anatomy which makes it difficult to develop and formulate effective drugs targeting brain to treat CNS disorders. But main barrier of the failure of developing effective CNS drug is the Blood-brain barrier (BBB) which hinders the delivery of drugs to the brain. Additionally, neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's diseases. But successful treatment plans and strategies are very limited due to the poor permeability of the drugs because of the presence of BBB. So, rather than developing a drug, distribution of drug to the target site by overcoming the BBB is the major concern of formulating CNS drugs (Patel et al., 2013). The BBB is highly selective towards what can enter the brain and what cannot by strictly regulating the transportation of solutes and cells (Upadhyay, 2014). Thus, BBB restricts the delivery of therapeutic substances to the brain and causes obstruction of many drugs such as neuropeptides. Because of the strict selectivity, BBB is one of the major reasons for poor drug delivery to the brain. Though molecules like glucose or fat soluble drugs can cross the brain, many drugs face difficulty because of fat-insoluble nature. Along with poor permeation properties, sometimes drugs bind to nontransporting proteins which result lesser drug absorption in brain cells. Furthermore, catabolic enzymes present in brain tissues can change the active form of drug making it inactive and non-functional (Upadhyay, 2014).

5.2 Different treatment strategies and Cell-penetrating peptides

Diverse methods, both invasive and non-invasive, have been developed to bypass the BBB and enhance brain medicine absorption (Kasinathan et al., 2014). A primary disadvantage of invasive methods (Table 3) is the trauma they create, in addition to their low therapeutic effectiveness and considerable negative effects. Non-invasive techniques, on the other hand, provide a number of advantages owing to their ability to retain the BBB while simultaneously increasing the peptides' permeability, stability, bioavailability, and/or receptor affinity. The peptide has been modified chemically, conjugated to shuttle molecules, and encapsulated in a non-invasive carrier. Among these efforts, peptide-vector-mediated delivery strategies, such as cell-penetrating peptides, have gained the most attention (CPPs). In recent decades, scientists have investigated CPPs, a type of naturally occurring, small peptides capable of translocating across cellular membranes (Ramsey & Flynn, 2015).

Table 3: Different treatment strategies and their limitations (Dong, 2018) (Teleanu et al., 2022)

Procedures	Strategies	Limitations
1. Invasive	Blood–brain barrier disruption	Not target specific, whole BBB disrupted
	Intracerebroventricular and intrathecal infusion	Administration by direct injection to brain, safety concerns
2. Non-invasive	Virus-mediated blood–brain barrier delivery	Administration by direct injection to brain, safety concerns
	Exosome-mediated delivery	Toxicity found in-vivo
	Solid-lipid nanoparticles	For smaller drug, administration, stability and storage concerns
	Modulating blood–brain barrier permeability	Incompatible findings in rodents and humans
	Liposome-based	Not currently used

Protein translocation domains (PTDs), membrane translocation sequences, and Trojan horse peptides are synonyms for CPPs. CPPs are a diverse set of peptides that typically include between 5 and 40 amino acids (AA). Without engaging with receptors, CPPs may traverse the tissues and membranes of mammals, plants, and microorganisms through energy-dependent or energy-independent pathways. Due to their capacity to increase the cellular internalization of covalently or non-covalently attached payloads while exhibiting low cytotoxicity in most cases, CPPs have generated considerable attention in the drug delivery industry (Pescina et al., 2018). CPPs are a viable method for transporting macromolecules across the BBB due to their powerful ability to facilitate fast passage across cell membranes. At addition, they are beneficial for treating CNS disorders because they prevent being filtered out by the P-glycoprotein (P-gp) in the blood-brain barrier (BBB) (Y. Zhang et al., 2021).

5.3 Internalization of CPPs

Despite the well-established fact that CPPs can carry a broad variety of payloads into cells, there is much dispute over the cell absorption processes, especially in terms of the desired goals of high targeting efficiency and low toxicity. CPP features (e.g., charge, structure, concentration, and length), cell types (e.g., membrane lipid composition, cell surface sugars, and peptide-to-lipid ratio), and cargo quality (size, type and charge) are often cited as reasons for the complexity of the transduction process. Many other (single or multiple) characteristics may influence membrane translocation processes because of CPP and CPP-cargo conjugate types and experimental conditions (pH, concentration and temperature). In Hela cell uptake experiments, for instance, when the concentration of R9 was increased from 5 M to 10 M, the mechanism moved from endocytosis to direct transport. Similar results were seen in investigations using TAT, with higher TAT concentrations often resulting in internalization of cells by direct translocation (Xu et al., 2019).Though CPPs have been used to research in

various fields, still proper translocation process is still unclear. Endocytosis and direct penetration are renowned mechanisms (Kwon et al., 2015).

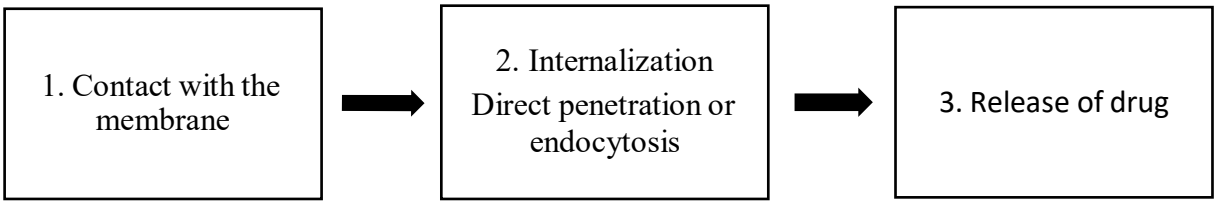

Figure 4: Steps of uptake of a cargo (Kardani et al., 2019).

Cargo-carrying CPPs are taken up by cells in three separate ways (Figure 4), irrespective of the kind of CPP or the targeted cell type. Electron transfer between peptides and negatively charged glycosaminoglycans (GAG) such as heparin, heparan sulfate, chondroitin sulfate, and proteoglycans on the cell surface promotes contact between cationic CPPs and anionic phospholipid bilayers (Xu et al., 2019). Two very different amphipathic and hydrophobic peptides rely on hydrophobic interactions with membranes; Wimley defined CPPs as "interfacially active peptides" for their ability to bind at the bilayer-water interface and perturb membrane structure by selectively activating some small GTPases, resulting in the remodeling of the action network and the formation of lamellipodia, and thus, contact with membrane is obtained (Pescina et al., 2018).

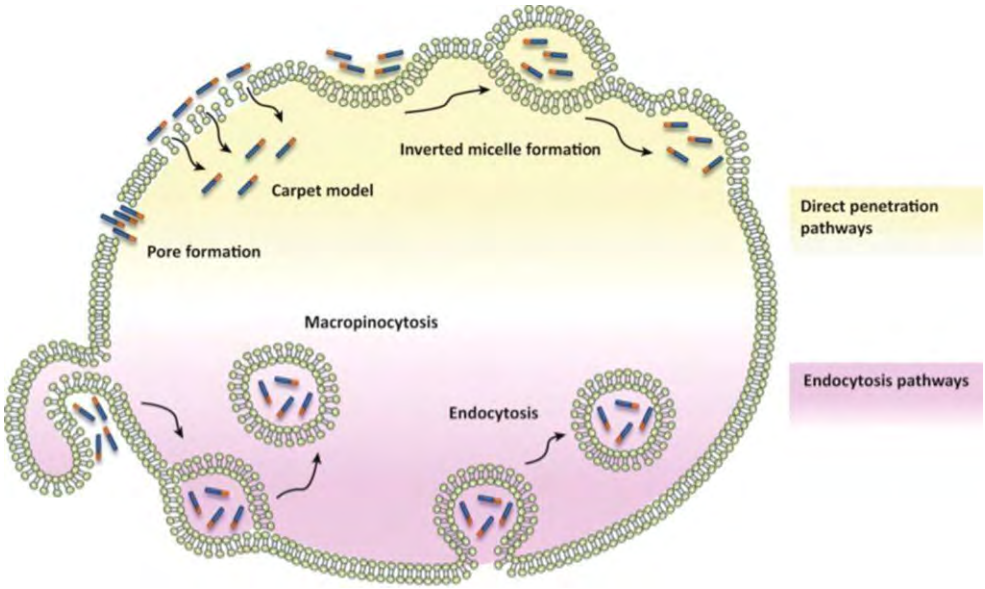

Figure 5: Schematic representation of proposed mechanisms for cell-penetrating peptides (CPPs) endocytosis(purple) and direct penetration(yellow) (Y. Zhang et al., 2021).

There are two naturally occurring forms of endocytosis: phagocytosis and pinocytosis. Phagocytosis is the mechanism by which only certain kinds of cells can absorb large particles (macrophages, monocytes and neutrophils). Nevertheless, all types of cells participate in a process known as pinocytosis (Figure 5), which involves the uptake of fluids and solutes (Ruseska & Zimmer, 2020). Endocytosis is triggered by electrostatic interactions with the cell surface proteoglycans or direct involvement with the plasma membrane. CPPs may enter cells when bound to glycosaminoglycans (GAGs) because GAGs undergo a continuous cycle of endocytosis and recycling, or CPPs can promote endocytosis by aggregating GAGs, activating intracellular signals, and altering actin (Graslund et al., 2011). The pinocytosis can also be catagorized as macropinocytosis. Because of the inward folding of the plasma membrane's outer surface, macropinosomes are generated during the process of macropinocytosis. The membrane of the generated macropinosomes is extremely similar to that of a cell membrane. Dynamin protein deficiency hinders membrane invagination. For

uptake, receptor-mediated endocytosis employs clathrin or caveolin pits. Clathrin and caveolin are the proteins that coat the intracellular membrane. After the extracellular molecule attaches to the membrane receptor, these molecules are required to invade the membrane and generate vesicles. Therefore, endocytosis is advantageous for bringing CPPs into the cell. However, how CPPs really leave the endosome is not known (Bechara & Sagan, 2013).

Direct penetration is an energy- and temperature-independent strategy including three basic mechanisms (Figure 6): pore formation, inverted micelle formation, and the carpet-like model. An early investigation shown that TAT is effective at both 4°C and 37° C. All of these procedures begin with CPP interacting with the membrane's negatively charged heparan sulfate (HS) and phospholipid bilayer. The folding of the peptide on the lipid membrane causes the membrane to become destabilized. Following internalization is determined by the lipid composition, peptide concentration, and peptide sequence of each model membrane. At high CPP concentrations, primary amphiphatic CPPs like transportan analogues and MPG are more likely to enter directly (Bechara & Sagan, 2013).In the second stage of absorption, peptide sequence, dose, and the lipid makeup of the cell membrane all play a role. CPPs, especially primary amphipathic peptides (e.g., MPG or transportan), may directly enter the cell membrane at high concentrations (Kardani et al., 2019) (Graslund et al., 2011) Kardani et al., 2019) (Xie et al., 2020).

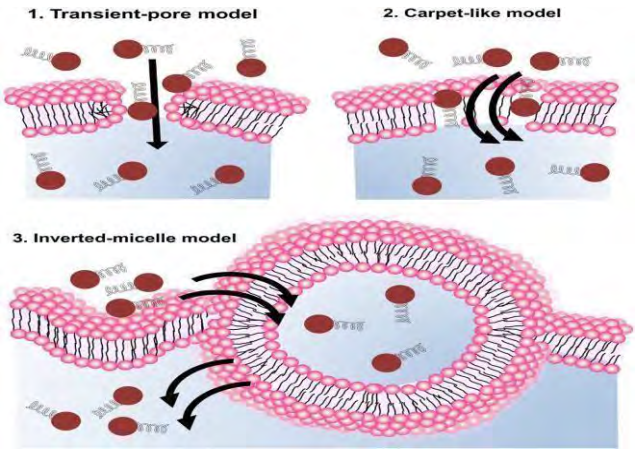

Figure 6: Schematic representation of direct penetration of CPPs (Nam et al., 2023).

This interaction between the positive side chains and phosphate groups is important for pore formation. Peptides accumulate in the outer leaflet between the phosphate and carbon chains of the lipids. Peptide accumulation thins the bilayer, and the attraction of arginine and lysine side chains to the proximal layer's headgroups provides a brief gap through which additional peptides diffuse, carrying with them the attached phospholipids. This shows that longer side chains on arginine residues are more advantageous for insertion and translocation than shorter side chains on lysine residues (Bechara & Sagan, 2013). In Figure 6, when an electrostatic bond is established between a peptide and the cellular membrane, the interaction alters the supramolecular structure of the lipids. As a consequence of this process, the membrane's curvature may change. When the membrane is bent or invaginated, inverted micelles, which trap the peptide, may form. The hydrophilic environment inside the inverted micelle facilitates both the accumulation of peptides and the transport of hydrophilic molecules bound to the peptide. The peptide-cargo combination is subsequently released into the cytoplasm upon destabilization of the micelle. The internalization occurs either by direct translocation or endocytosis, depending on penetratin concentration. In Figure 4, the interaction between cationic CPPs (such as Tat peptide at high concentrations) and negatively charged phospholipid led to membrane thinning and carpeting (Graslund et al.,2011).

5.4 Secondary structure of CPPs and Nuclear magnetic resolution (NMR):

CPPs when interact with cellular membrane, they tend to attain a secondary structure (Eiríksdóttir et al., 2010). Most CPPs tend to attain amphiphilic α-helical structure but shows a chameleon-like characteristics that they are ready to change their structure according to the membrane environment. Electrostatic interaction between positive charge of CPPs and negatively charge cell membrane can cause secondary structure formation. This behavior can influence how the CPPs will act on cellular membrane (Mäler, 2013). There are different methods to analyze the interaction of peptides and cellular membrane (Table 2). Interaction ofCPPs with lipid bilayer and the conversion into secondary structure is mostly analyzed by using nuclear magnetic resolution (NMR). Because NMR not only indentifies the structure of macromolecules but also can analyzes their interaction (Zorko & Langel, 2022). Then, interaction in biological cellular pathways can be analyzed by using NMR with the help of cell line (Coronado et al., 2022). Generally, solution based NMR studies are performed to analyze the interaction of CPPs (Mäler, 2012). RW16, a multi-purpose CPPs, has been analyzed by using NMR (Jobin et al., 2019).

Table 4: Secondary structure visualization methods (Neundorf, 2019).

Methods	Application
Infrared(IR) spectroscopy	To analyze secondary structures of peptides in the presence of lipid phases
Circular dichroism (CD) spectroscopy	To analyze secondary structures of peptides in the presence of lipid phases , membrane vesicles or bacteria
Methods	Applications
Nuclear magnetic resonance (NMR) spectroscopy	To analyze three-dimensional(3D) structure of peptides in lipid phases by the combination of artificial cell membrane and solid or solution NMR

5.5 Intranasal drug delivery to brain

Oral medicine delivery is the least invasive option in terms of patient comfort and safety. However, oral delivery is challenging for protein and peptide distribution due to enzymatic degradation and limited intestinal mucosal permeability (Khafagy et al., 2009). However, intracranial injection is a direct but difficult approach for administering drugs to the brain. For drugs such as biomacromolecular pharmaceuticals that cannot cross the BBB, intracranial injection is the only treatment option, notwithstanding the high risk associated with surgical operations. In addition to the BBB, however, the considerably more difficult problem of drug diffusion across CNS compartments must be addressed. Due to their hydrophilic nature and large size, protein distribution is restricted to injection sites, despite the fact that intra

parenchymal or CSF administration may provide a high degree of targeting. Due to the BBB and intracerebral diffusion, efforts to provide protein-based medications to the brain are often futile. Consequently, research into innovative techniques for crossing the BBB and delivering drugs to the CNS remains a vital need. Due to its ability to circumvent the BBB and its accessibility, the transport of medications via the nose passages through the olfactory axonal pathway from the epithelium into cerebral tissue has piqued scientific attention. There is evidence that olfactory axonal transport transports proteins to the central nervous system. The natural CPP, low molecular weight protamine (LMWP) has been shown (Figure 7) to facilitate transit from the nose to the brain (Lin et al., 2016). For gliomas, cancer cells spread all over the brain. Conventional treatments like radiotherapy, chemotherapy, anti-cancer drugs have poor therapeutic efficacy. For this, siRNA (Small Interfering Ribonucleic acid) loaded in PEG-PCL-Tat CPPs has been designed for nose to brain delivery (Kanazawa et al., 2020). PEG-PCL-Tat CPPs has been proved efficient for delivering siRNA into the brain by intranasal routes (Kurano et al., 2022).

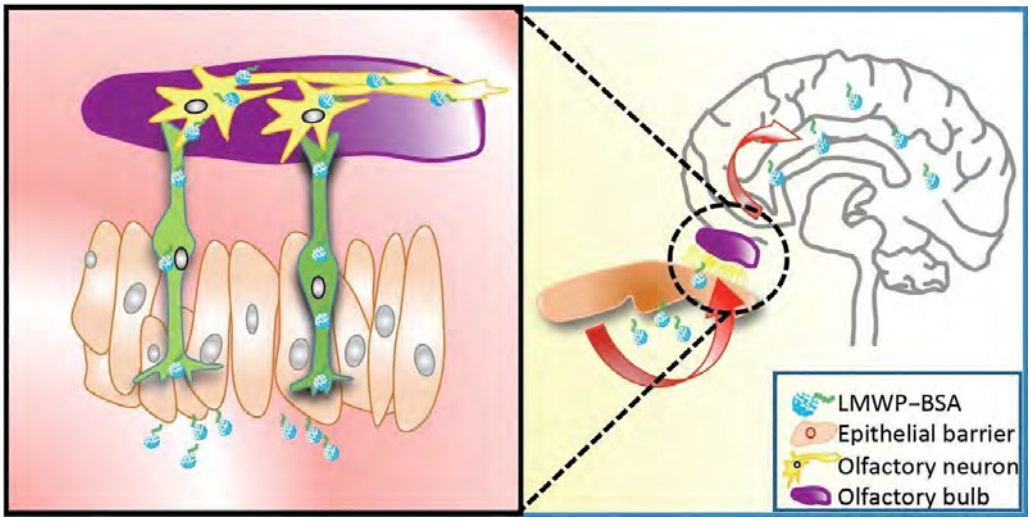

Figure 7: The cell-penetrating LMWP peptide-mediated protein drug from nose to brain delivery (Lin et al., 2016)

5.6 Clinical studies on drug delivery to CNS by using CPP

Genetically modified adeno-associated virus (rAAV) vectors are also seen as a potential platform for the treatment of CNS disorders. Due to the difficulty of AAV vectors to successfully cross the blood-brain barrier (BBB), intraparenchymal injection or high-dose system administration is required for the treatment of CNS disorders using AAV-mediated gene therapy. However, the majority of neurodegenerative illnesses, including as amyotrophic lateral sclerosis, Huntington's disease, leukodystrophies, and lysosomal storage diseases, involve cell destruction at various places across the brain, rendering intraparenchymal injection of AAV vectors useless. CPP has thus been investigated as a way of delivering AAV into the cell. Certain AAV serotypes may transduce neurons and other types of brain cells; the most successful of them is AAV9. AAV9 can transduce neurons, parenchymal brain cells, and even a portion of the BBB endothelium. The findings of this research were published in (Meng et al., 2021) (Haery et al., 2019). On a laboratory plate, the tests were performed.

5.6.1 CPPs mediated enhanced transduction of AAV9 in endothelial cells (ECs) and human astrocytes (HAs):

The majority of the BBB is composed of microvascular endothelial cells (ECs) and astrocytes. ECs in the brain's microvasculature serve a function in regulating the amount of a chemical that enters and exits the brain, while astrocytes are essential for the formation and maintenance of the blood-brain barrier. Astrocytes, the most common kind of glial cell in the human brain, provide nutritional and metabolic support to their CNS neuronal partners in addition to developmental guidance. After infecting human cerebral microvascular ECs and Hashi cells with an AAV9 vector expressing green fluorescent protein (GFP) to assess the

impact of CPPs on AAV9 transduction in human cells, the transduction efficiency was determined (Meng et al., 2021).

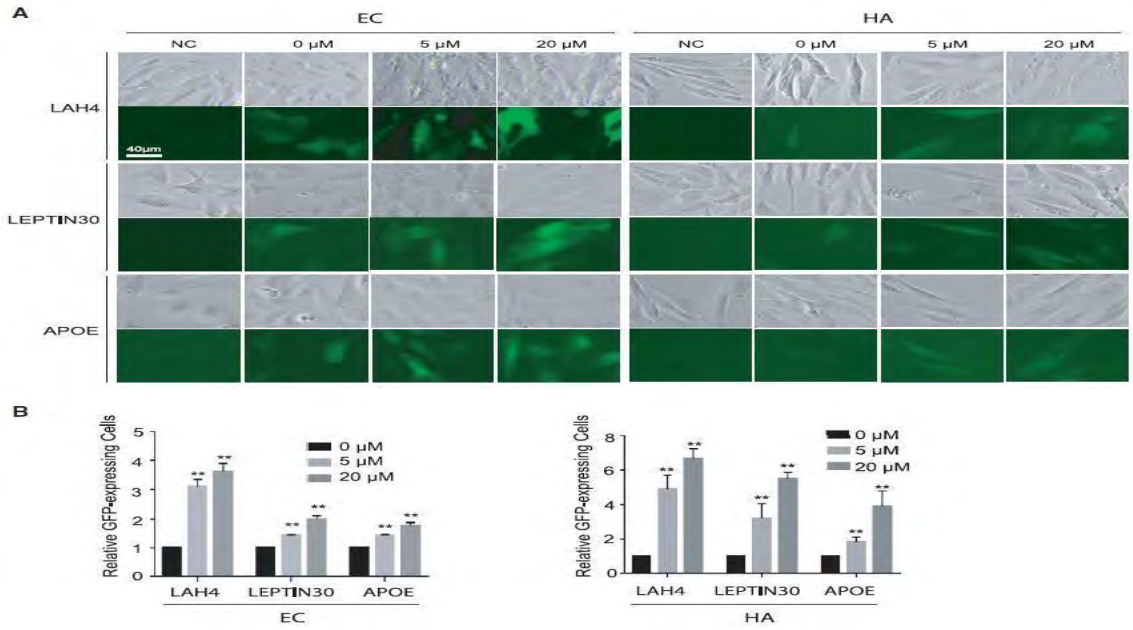

Figure 8: (A) LAH4, LEPTIN30, and APOE CPPs enhanced transduction of AAV9 to ECs and HAs. ECs or HAs were infected with AAV9/GFP (MOI of 1,000) alone or precomplexed without or with 5 or 20 mM LAH4, LEPTIN30, or APOE. (B) Quantification of GFP- expressing cellsin (A) (Meng et al., 2021).

AAV9 dosages were remained constant while CPP concentrations were increased from 0 to 5 to 20 mM. (MOI of 1,000). LAH4, LEPTIN30, and APOE improved the viral transduction efficiency of ECs and Has, as shown in Figures 8A and 8B. Negative control (NC) cells that had not been transduced with AAV9 lacked fluorescence. It was discovered that cells treated only with the AAV9 vector fluoresced very faintly. The number of GFP-expressing cells increased by 310%, 142%, and 140% when the AAV9 vector was pre-incubated with the peptide (5 mM) of LAH4, LEPTIN30, or APOE, followed by transduction with ECs or HAs. Compared to cells transduced with AAV alone, the number of GFP-expressing cells

increased by about 360%, 200%, and 170% when the concentration of CPPs was increased to 20 mM in ECs and HAs, respectively. LAH4 enhanced the viral transduction in ECs and HAs more than LEPTIN30 or APOE. All of these findings indicate that CPPs may significantly improve AAV9 transduction into ECs and HAs (Meng et al., 2021).

5.6.2 CPPs used for CNS

Table 5: Characteristics of CPPs that were selected for BBB transport property (Stalmans et al., 2015).

CPP	**Sequence**	**Molecular weight(Da)**	**Characteristic**	**Uptake mechanism**
pVEC	LLILRRRIRKQAHAHSK-NH_2	2208.8	Amphipathic-Cationic	Direct penetration and/Endocytosis
TP10	AGYLLGKINLKALAALAKKIL-NH_2	2181.8	Amphipathic-Cationic	Direct penetration and/Endocytosis
TP10-2	AGYLLGKINLKPLAALAKKL-NH_2	2207.8	Amphipathic-Cationic	Direct penetration
SynB3	RRLSYSRRRF- NH_2	1396.7	Cationic	Endocytosis
Tat 47-57	YGRKKRRCRRR- NH_2	1568.9	Cationic	Direct penetration and/Endocytosis

Chapter 6

Discussion

The BBB is the most essential of multiple barrier structures that protect the central nervous system from the effects of exogenous medicines while permitting the passage of endogenous substances. Because of this, it is very difficult to deliver drugs to brain lesions in a safe and effective manner. Finding a secure, trustworthy, and effective technique to bypass the BBB is an urgent matter that must be addressed quickly. CPPs have therapeutic promise as medications, vectors, and ligands in the delivery system for treating CNS disorders. The disadvantages of traditional techniques of delivering medications to the brain are many. Surgeries, intravenous drug delivery are highly invasive resulting traumas in our brain. But still, the drugs cannot reach properly to the target sites causing more side effects rather than therapeutic efficacy. Besides, invasive approaches causes BBB disruption which can aggregates the neurodegenerative disorders. CPPs, on the other hand, transport molecules with a high degree of penetration. They can very effectively attach to the cell surface in an almost endless number of instances, and have negligible cytotoxicity. CPPs considerably promote the in vitro and in vivo delivery of physiologically active molecules, such as small molecules, proteins, nucleic acids, and nanoparticles, because to their propensity for subcellular localization, which increases the intracellular trafficking of transported compounds. This has been proven many studies. So, to overcome the barrier of BBB, using CPPs to deliver cargo can be a better approach to deliver the drugs for CNS disorders.

Chapter 7

Limitations and approaches on how to overcome

While cationic and hydrophobic CPPs are able to transduce a wide variety of tissues in vivo, their lack of cell selectivity raises the likelihood of off-target, non-specific effects, which may result in undesired side effects (Zahid & Robbins, 2015). The overwhelming majority of first-generation CPPs lack selectivity for individual cells. The membranes of mammalian cells and non-mammalian cells vary significantly. In contrast, the CPPs lack selectivity in the cells, tissues, and organs of the same organism. Varying cell types have very different susceptibilities for CPPs, auxiliaries, and cargos, while having equal absorption efficiencies (Reissmann, 2014). Cell penetrating peptides are peptides that can penetrate cells but are generally inhibited by their membranes (CPPs). CPP-cargo conjugates specifically exhibit a predisposition for endocytosis-mediated accumulation inside cells. However, they have difficulty leaving endocytic organelles and entering the cytoplasm of cells, where they can perform their functions. Consequently, the efficacy of CPP is lowered if they cannot undergo endosomal escape. In addition, nothing is known regarding the mechanism of endosomal escpae (Nam et al., 2023).While a healthy human body has a pH of 7.4, an inflammatory lesion or cancerous location may have a pH in the more acidic range (5.5–6.0). It is usual practice to maximize cellular absorption of CPPs at a predefined pH to selectively deliver the cargo of interest and to promote successful endosomal escape. Histidine, one of the amino acids that comprise CPPs, has a cationic charge at pH 6.0 or lower but is neutral at the normal pH 7.4. Histidine-modified CPPs for pH-responsive drug delivery have been developed (Nam et al., 2023). Enhanced cell selectivity is a further advantage of liposomal nanocarriers (Reissmann, 2014). Activatable cell penetrating peptides (ACPPs) have been used extensively in tumor therapy and molecular imaging probes due to their unique control mechanism based on the selective and local release of CPP. ACPPs may target matrix

metalloproteinases (MMPs), thrombin, and lysozyme via the controlled release mechanism of a well constructed linker (D. Zhang et al., 2016).

Chapter 8

Future aspects and conclusion

Fibroblast peptide research is a rapidly increasing field of study. These peptides may transport antibodies, enzymes, chaperones, substrates, inhibitors, and nucleic acids including nucleoside phosphates, oligonucleotides, mimics of them, interfering RNA, and DNA into live cells. Consequently, they may be used as tools in the domains of cellular biology and signal transduction/intracellular signaling research. CPPs are more therapeutically promising than electroporation, magnetofection, lipidofection, and viral vectors. Due to their low toxicity in mammalian cells, these agents may be used to distribute poorly permeable or impermeable generic medications via the skin, conjunctiva of the eyes, and blood-brain barrier. As a noninvasive means of providing medicine to the brain, CPPs also reduces the hazards associated with intravenous drug administration. In addition, further study is necessary to grasp CPPs and their activities inside the human body. In conclusion, CPPs provide interesting applications in the field of brain-targeted drug delivery.

References

Antoniou, X., & Borsello, T. (2010). Cell permeable peptides: A promising tool to deliver neuroprotective agents in the brain. *Pharmaceuticals*, *3*(2), 379–392. https://doi.org/10.3390/ph3020379

Arib, C., Griveau, A., Eyer, J., & Spadavecchia, J. (2022). Cell penetrating peptide (CPP) gold(iii) - complex - bioconjugates: from chemical design to interaction with cancer cells for nanomedicine applications. *Nanoscale Advances*, *4*(14), 3010–3022. https://doi.org/10.1039/d2na00096b

Backlund, C. M., Holden, R. L., Moynihan, K. D., Garafola, D., Farquhar, C., Mehta, N. K., Maiorino, L., Pham, S., Iorgulescu, J. B., Reardon, D. A., Wu, C. J., Pentelute, B. L., & Irvine, D. J. (2022). Cell-penetrating peptides enhance peptide vaccine accumulation and persistence in lymph nodes to drive immunogenicity. *Proceedings of the National Academy of Sciences of the United States of America*, *119*(32). https://doi.org/10.1073/pnas.2204078119

Bartlett, R. L., Sharma, S., & Panitch, A. (2013). Cell-penetrating peptides released from thermosensitive nanoparticles suppress pro-inflammatory cytokine response by specifically targeting inflamed cartilage explants. *Nanomedicine: Nanotechnology, Biology, and Medicine*, *9*(3), 419–427. https://doi.org/10.1016/j.nano.2012.09.003

Boisguérin, P., Deshayes, S., Gait, M. J., O'Donovan, L., Godfrey, C., Betts, C. A., Wood, M. J. A., & Lebleu, B. (2015). Delivery of therapeutic oligonucleotides with cell penetrating peptides. *Advanced Drug Delivery Reviews*, *87*, 52–67. https://doi.org/10.1016/j.addr.2015.02.008

Borrelli, A., Tornesello, A. L., Tornesello, M. L., & Buonaguro, F. M. (2018). Cell penetrating peptides as molecular carriers for anti-cancer agents. *Molecules*, *23*(2).

https://doi.org/10.3390/molecules23020295

Chen, W., Hu, Y., & Ju, D. (2020). Gene therapy for neurodegenerative disorders: advances, insights and prospects. *Acta Pharmaceutica Sinica B*, *10*(8), 1347–1359. https://doi.org/10.1016/j.apsb.2020.01.015

Coronado, M. A., de Moraes, F. R., Stuqui, B., Calmon, M. F., Eberle, R. J., Rahal, P., & Arni, R. K. (2022). The Secreted Metabolome of Hela Cells under Effect of Crotamine, a Cell-Penetrating Peptide from a Rattlesnake Using NMR-Based Metabolomics Analyses. *BioMed*, *2*(2), 238–254. https://doi.org/10.3390/biomed2020020

Davie, C. A. (2008). A review of Parkinson's disease. *British Medical Bulletin*, *86*(1), 109–127. https://doi.org/10.1093/bmb/ldn013

Delcroix, M., & Riley, L. W. (2010). Cell-penetrating peptides for antiviral drug development. *Pharmaceuticals*, *3*(3), 448–470. https://doi.org/10.3390/ph3030448

Dong, X. (2018). Current strategies for brain drug delivery. *Theranostics*, *8*(6), 1481–1493. https://doi.org/10.7150/thno.21254

Eiríksdóttir, E., Konate, K., Langel, Ü., Divita, G., & Deshayes, S. (2010). Secondary structure of cell-penetrating peptides controls membrane interaction and insertion. *Biochimica et Biophysica Acta - Biomembranes*, *1798*(6), 1119–1128. https://doi.org/10.1016/j.bbamem.2010.03.005

Foged, C., & Nielsen, H. M. (2008). *Cell-penetrating peptides for drug delivery across membrane barriers*. 105–118.

Frøslev, P., Franzyk, H., Ozgür, B., Brodin, B., & Kristensen, M. (2022). Highly cationic cell-penetrating peptides affect the barrier integrity and facilitates mannitol permeation in a human stem cell-based blood-brain barrier model. *European Journal of*

Pharmaceutical Sciences, *168*(August 2021). https://doi.org/10.1016/j.ejps.2021.106054

Fu, T. K., Kuo, P. H., Lu, Y. C., Lin, H. N., Wang, L. H. C., Lin, Y. C., Kao, Y. C., Lai, H. M., & Chang, M. D. T. (2020). Cell penetrating peptide as a high safety anti-inflammation ingredient for cosmetic applications. *Biomolecules*, *10*(1). https://doi.org/10.3390/biom10010101

Gessner, I., & Neundorf, I. (2020). Nanoparticles modified with cell-penetrating peptides: Conjugation mechanisms, physicochemical properties, and application in cancer diagnosis and therapy. *International Journal of Molecular Sciences*, *21*(7), 1–21. https://doi.org/10.3390/ijms21072536

Guo, Z., Peng, H., Kang, J., & Sun, D. (2016). Cell-penetrating peptides: Possible transduction mechanisms and therapeutic applications (review). *Biomedical Reports*, *4*(5), 528–534. https://doi.org/10.3892/br.2016.639

Gupta, S., Dhanda, S., & Sandhir, R. (2018). Anatomy and physiology of blood-brain barrier. In *Brain Targeted Drug Delivery Systems: A Focus on Nanotechnology and Nanoparticulates* (Issue c). Elsevier Ltd. https://doi.org/10.1016/B978-0-12-814001-7.00002-0

Habault, J., & Poyet, J. L. (2019). Recent advances in cell penetrating peptide-based anticancer therapies. *Molecules*, *24*(5), 1–17. https://doi.org/10.3390/molecules24050927

Hasannejad-Asl, B., Pooresmaeil, F., Takamoli, S., Dabiri, M., & Bolhassani, A. (2022). Cell penetrating peptide: A potent delivery system in vaccine development. *Frontiers in Pharmacology*, *13*(November), 1–17. https://doi.org/10.3389/fphar.2022.1072685

Jobin, M. L., Blanchet, M., Henry, S., Chaignepain, S., Manigand, C., Castano, S., Lecomte,

S., Burlina, F., Sagan, S., & Alves, I. D. (2015). The role of tryptophans on the cellular uptake and membrane interaction of arginine-rich cell penetrating peptides. *Biochimica et Biophysica Acta - Biomembranes*, *1848*(2), 593–602. https://doi.org/10.1016/j.bbamem.2014.11.013

Jobin, M. L., Vamparys, L., Deniau, R., Grélard, A., Mackereth, C. D., Fuchs, P. F. J., & Alves, I. D. (2019). Biophysical insight on the membrane insertion of an arginine-rich cell-penetrating peptide. *International Journal of Molecular Sciences*, *20*(18). https://doi.org/10.3390/ijms20184441

Kanazawa, T., Taki, H., & Okada, H. (2020). Nose-to-brain drug delivery system with ligand/cell-penetrating peptide-modified polymeric nano-micelles for intracerebral gliomas. *European Journal of Pharmaceutics and Biopharmaceutics*, *152*(May), 85–94. https://doi.org/10.1016/j.ejpb.2020.05.001

Kasinathan, N., Jagani, H. V, Alex, A. T., Volety, S. M., & Rao, J. V. (2014). *Strategies for drug delivery to the central nervous system by systemic route*. *7544*, 1–15. https://doi.org/10.3109/10717544.2013.878858

Kebebe, D., Liu, Y., Wu, Y., Vilakhamxay, M., Liu, Z., & Li, J. (2018). Tumor-targeting delivery of herb-based drugs modified nanocarriers. *International Journal of Nanomedicine*, 1425–1442.

Koren, E., & Torchilin, V. P. (2012). Cell-penetrating peptides: breaking through to the other side. *Trends in Molecular Medicine*, *18*(7), 385–393. https://doi.org/10.1016/j.molmed.2012.04.012

Kristensen, M., Birch, D., & Nielsen, H. M. (2016). Applications and challenges for use of cell-penetrating peptides as delivery vectors for peptide and protein cargos. *International Journal of Molecular Sciences*, *17*(2).

https://doi.org/10.3390/ijms17020185

Kumar Thakur, A., Kamboj, P., Goswami, K., & Ahuja, K. (2018). Pathophysiology and management of alzheimer's disease: an overview. *Journal of Analytical & Pharmaceutical Research*, *7*(2), 226–235. https://doi.org/10.15406/japlr.2018.07.00230

Kurano, T., Kanazawa, T., Iioka, S., Kondo, H., Kosuge, Y., & Suzuki, T. (2022). Intranasal Administration of N-acetyl-L-cysteine Combined with Cell-Penetrating Peptide-Modified Polymer Nanomicelles as a Potential Therapeutic Approach for Amyotrophic Lateral Sclerosis. *Pharmaceutics*, *14*(12). https://doi.org/10.3390/pharmaceutics14122590

Kurrikoff, K., Gestin, M., & Langel, Ü. (2016). Recent in vivo advances in cell-penetrating peptide-assisted drug delivery. *Expert Opinion on Drug Delivery*, *13*(3), 373–387. https://doi.org/10.1517/17425247.2016.1125879

Kwon, S. S., Kim, S. Y., Kong, B. J., Kim, K. J., Noh, G. Y., Im, N. R., Lim, J. W., Ha, J. H., Kim, J., & Park, S. N. (2015). Cell penetrating peptide conjugated liposomes as transdermal delivery system of Polygonum aviculare L. extract. *International Journal of Pharmaceutics*, *483*(1–2), 26–37. https://doi.org/10.1016/j.ijpharm.2015.01.030

Lin, T., Liu, E., He, H., Shin, M. C., Moon, C., Yang, V. C., & Huang, Y. (2016). Nose-to-brain delivery of macromolecules mediated by cell-penetrating peptides. *Acta Pharmaceutica Sinica B*, *6*(4), 352–358. https://doi.org/10.1016/j.apsb.2016.04.001

Liu, Y., Zhao, Z., & Li, M. (2022). Overcoming the cellular barriers and beyond: Recent progress on cell penetrating peptide modified nanomedicine in combating physiological and pathological barriers. *Asian Journal of Pharmaceutical Sciences*, *17*(4), 523–543. https://doi.org/10.1016/j.ajps.2022.05.002

Mäler, L. (2012). Solution NMR studies of peptide-lipid interactions in model membranes. *Molecular Membrane Biology*, *29*(5), 155–176. https://doi.org/10.3109/09687688.2012.683456

Mäler, L. (2013). Solution NMR studies of cell-penetrating peptides in model membrane systems. *Advanced Drug Delivery Reviews*, *65*(8), 1002–1011. https://doi.org/10.1016/j.addr.2012.10.011

Meng, Y., Sun, D., Qin, Y., Dong, X., Luo, G., & Liu, Y. (2021). Cell-penetrating peptides enhance the transduction of adeno-associated virus serotype 9 in the central nervous system. *Molecular Therapy - Methods and Clinical Development*, *21*(June), 28–41. https://doi.org/10.1016/j.omtm.2021.02.019

Morén, C., deSouza, R. M., Giraldo, D. M., & Uff, C. (2022). Antioxidant Therapeutic Strategies in Neurodegenerative Diseases. *International Journal of Molecular Sciences*, *23*(16). https://doi.org/10.3390/ijms23169328

Mortada, I., Farah, R., Nabha, S., Ojcius, D. M., Fares, Y., Almawi, W. Y., & Sadier, N. S. (2021). Immunotherapies for Neurodegenerative Diseases. *Frontiers in Neurology*, *12*(June), 1–19. https://doi.org/10.3389/fneur.2021.654739

Mussa Farkhani, S., Asoudeh Fard, A., Zakeri-Milani, P., Shahbazi Mojarrad, J., & Valizadeh, H. (2017). Enhancing antitumor activity of silver nanoparticles by modification with cell-penetrating peptides. *Artificial Cells, Nanomedicine and Biotechnology*, *45*(5), 1029–1035. https://doi.org/10.1080/21691401.2016.1200059

Nam, S. H., Park, J., & Koo, H. (2023). Recent advances in selective and targeted drug/gene delivery systems using cell-penetrating peptides. *Archives of Pharmacal Research*, *46*(1), 18–34. https://doi.org/10.1007/s12272-022-01425-y

Neundorf, I. (2019). Antimicrobial and cell-penetrating peptides: How to understand two distinct functions despite similar physicochemical properties. *Advances in Experimental Medicine and Biology*, *1117*, 93–109. https://doi.org/10.1007/978-981-13-3588-4_7

Patel, M., Souto, E. B., & Singh, K. K. (2013). Advances in brain drug targeting and delivery: limitations and challenges of solid lipid nanoparticles. *Expert Opinion on Drug Delivery*, *10*(7), 889–905. https://doi.org/10.1517/17425247.2013.784742

Ramsey, J. D., & Flynn, N. H. (2015). Cell-penetrating peptides transport therapeutics into cells. *Pharmacology and Therapeutics*, *154*, 78–86. https://doi.org/10.1016/j.pharmthera.2015.07.003

Riva, A., Golda, A., Balagura, G., Amadori, E., Vari, M. S., Piccolo, G., Iacomino, M., Lattanzi, S., Salpietro, V., Minetti, C., & Striano, P. (2021). New Trends and Most Promising Therapeutic Strategies for Epilepsy Treatment. *Frontiers in Neurology*, *12*(December), 1–14. https://doi.org/10.3389/fneur.2021.753753

Sadeghian, I., Heidari, R., Sadeghian, S., Raee, M. J., & Negahdaripour, M. (2022). Potential of cell-penetrating peptides (CPPs) in delivery of antiviral therapeutics and vaccines. *European Journal of Pharmaceutical Sciences*, *169*(September 2021). https://doi.org/10.1016/j.ejps.2021.106094

Serlin, Y., Shelef, I., Knyazer, B., & Friedman, A. (2015). Anatomy and physiology of the blood-brain barrier. *Seminars in Cell and Developmental Biology*, *38*, 2–6. https://doi.org/10.1016/j.semcdb.2015.01.002

Stalmans, S., Bracke, N., Wynendaele, E., Gevaert, B., Peremans, K., Burvenich, C., Polis, I., & De Spiegeleer, B. (2015). Cell-penetrating peptides selectively cross the blood-brain barrier in vivo. *PLoS ONE*, *10*(10), 1–22. https://doi.org/10.1371/journal.pone.0139652

Tabujew, I., Lelle, M., & Peneva, K. (2015). Cell-penetrating peptides for nanomedicine-how to choose the right peptide. *BioNanoMaterials*, *16*(1), 59–72. https://doi.org/10.1515/bnm-2015-0001

Teleanu, R. I., Preda, M. D., Niculescu, A. G., Vladâcenco, O., Radu, C. I., Grumezescu, A. M., & Teleanu, D. M. (2022). Current Strategies to Enhance Delivery of Drugs across the Blood–Brain Barrier. *Pharmaceutics*, *14*(5), 1–30. https://doi.org/10.3390/pharmaceutics14050987

Tripathi, P. P., Arami, H., Banga, I., Gupta, J., & Gandhi, S. (2018). Cell penetrating peptides in preclinical and clinical cancer diagnosis and therapy. *Oncotarget*, *9*(98), 37252–37267. https://doi.org/10.18632/oncotarget.26442

Uhl, P., Grundmann, C., Sauter, M., Storck, P., Tursch, A., Özbek, S., Leotta, K., Roth, R., Witzigmann, D., Kulkarni, J. A., Fidelj, V., Kleist, C., Cullis, P. R., Fricker, G., & Mier, W. (2020). Coating of PLA-nanoparticles with cyclic, arginine-rich cell penetrating peptides enables oral delivery of liraglutide. *Nanomedicine: Nanotechnology, Biology, and Medicine*, *24*, 102132. https://doi.org/10.1016/j.nano.2019.102132

Upadhyay, R. K. (2014). Drug delivery systems, CNS protection, and the blood brain barrier. *BioMed Research International*, *2014*. https://doi.org/10.1155/2014/869269

Wang, Y. F., Xu, X., Fan, X., Zhang, C., Wei, Q., Xi, W., Guo, W., Wei, X., Yu, J., Yan, J. L., & Liang, H. P. (2011). A cell-penetrating peptide suppresses inflammation by inhibiting NF-κB signaling. *Molecular Therapy*, *19*(10), 1849–1857. https://doi.org/10.1038/mt.2011.82

Wilhelm, I., Molnár, J., Fazakas, C., Haskó, J., & Krizbai, I. A. (2013). Role of the blood-brain barrier in the formation of brain metastases. *International Journal of Molecular Sciences*, *14*(1), 1383–1411. https://doi.org/10.3390/ijms14011383

Xie, J., Bi, Y., Zhang, H., Dong, S., Teng, L., Lee, R. J., & Yang, Z. (2020). Cell-Penetrating Peptides in Diagnosis and Treatment of Human Diseases: From Preclinical Research to Clinical Application. *Frontiers in Pharmacology*, *11*(May), 1–23. https://doi.org/10.3389/fphar.2020.00697

Yang, J., Luo, Y., Shibu, M. A., Toth, I., & Skwarczynskia, M. (2019). Cell-penetrating Peptides: Efficient Vectors for Vaccine Delivery. *Current Drug Delivery*, *16*(5), 430–443. https://doi.org/10.2174/1567201816666190123120915

Yuan, Q., Pan, A., Fu, Y., & Dai, Y. (2020). Anatomy and Physiology of the Blood–Brain Barrier. In *Integrative Pancreatic Intervention Therapy: A Holistic Approach*. Elsevier Inc. https://doi.org/10.1016/B978-0-12-819402-7.00001-2

Zahid, M., & Robbins, P. D. (2015). Cell-type specific penetrating peptides: Therapeutic promises and challenges. *Molecules*, *20*(7), 13055–13070. https://doi.org/10.3390/molecules200713055

Zhang, S., Gan, L., Cao, F., Wang, H., Gong, P., Ma, C., Ren, L., Lin, Y., & Lin, X. (2022). The barrier and interface mechanisms of the brain barrier, and brain drug delivery. *Brain Research Bulletin*, *190*(April), 69–83. https://doi.org/10.1016/j.brainresbull.2022.09.017

Zhang, Y., Guo, P., Ma, Z., Lu, P., Kebebe, D., & Liu, Z. (2021). Combination of cell-penetrating peptides with nanomaterials for the potential therapeutics of central nervous system disorders: a review. *Journal of Nanobiotechnology*, *19*(1), 1–22. https://doi.org/10.1186/s12951-021-01002-3

Zorko, M., & Langel, Ü. (2022). Studies of cell-penetrating peptides by biophysical methods. *Quarterly Reviews of Biophysics*, *55*. https://doi.org/10.1017/S0033583522000026