Delivery of Neurotherapeutics Across the Blood Brain Barrier in Stroke

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Abstract: Stroke is a devastating disease with few therapeutic options. Despite our growing understanding of the critical mechanistic events in post-stroke brain injury, the clinical translation of these findings has been less effective. A monumental hurdle to the field has been the inability of many systemically applied therapies to efficiently cross the blood brain barrier (BBB) and enter brain cells. Over the last two decades, however, significant technological achievements have overcome this obstacle to facilitate central nervous system (CNS) drug delivery. Noninvasive drug carriers, especially cell penetrating peptide (CPP) show great potential to deliver neurotherapeutics across the BBB for the treatment of ischemic brain injury. This review begins with a brief introduction to the BBB in relation to drug delivery and then provides an overview of the development of drug carriers for neurotherapeutics, with a focus on CPP-mediated transduction. We discuss recent advances and limitations in this field, as well as mechanisms underlying CPP-mediated brain targeting. We also summarize the application of CPPs in stroke research. Continuing modifications and improvements of CPPs are expected to enhance both their feasibility in clinical stroke management and their specificity towards particular cell types.

Keywords: Cell penetrating peptide, TAT protein transduction domains, blood brain barrier, stroke, neuroprotection.

INTRODUCTION

Stroke is one of the most common causes of death and the leading cause of serious long-term adult disability in the United States. Currently approved therapies for ischemic stroke are limited by a short temporal window of opportunity and low success rate. The development of new therapeutic strategies for stroke is imperative. In recent years, an increasing number of molecules have been identified as potential therapeutic agents for central nervous system (CNS) diseases such as stroke. However, the blood brain barrier (BBB) prevents the free passage of many of these substances into the CNS and has stymied drug development in this area. For example, nearly all peptide- or protein-based drugs fail to penetrate into brain tissue to an appreciable extent and have led to very few clinical applications. Thus, the development of drug delivery and targeting strategies to overcome the obstacle of the BBB is more urgent now than ever before. Here, we discuss recent developments in drug carriers for neurotherapeutics, with particular focus on cell penetrating peptide (CPP)-mediated transduction. We review the mechanisms, recent achievements, and limitations of CPP-mediated delivery and the application of CPP to stroke research.

BLOOD BRAIN BARRIER: AN OBSTACLE TO EFFECTIVE CNS DRUG DELIVERY

The presence of a barrier between the blood and the brain was first observed by Paul Ehrlich and Edwin Goldman a century ago with intravenous versus intracranial infusions of dyes such as Trypan Blue (for reviews see [1, 2]). However, it is Lewandowski that is often credited for researchers for coining the term “Bluthmishranke” (BBB in German) and Lina Stern for coining the term “hematoencephalic barrier” [1, 2]. Although there is also a blood-cerebrospinal fluid barrier, an arachnoid barrier, and a blood-retinal barrier, the study of the BBB for the past century has overshadowed that of all the others. As a whole, these studies confirm that the BBB is a highly effective physical and metabolic barrier segregating CNS parenchyma from the blood circulation. It prevents the passage of non-CNS cells, various foreign substances and microbes from the bloodstream to the CNS. As a result of its efficacy in protecting vulnerable post-mitotic neurons and resident glia from potentially harmful peripheral substances, it also acts as a natural obstacle for the systemic delivery of neurotherapeutics. The BBB is composed principally of specialized capillary endothelial cells sealed by highly restrictive tight junctions not found elsewhere in the circulation. These endothelial cells are, in turn, surrounded by pericytes that cover 32% of the brain capillaries [3]. Pericytes synthesize most elements of the basement membrane and share this membrane with the endothelial cells. Astrocytes embrace the outer surface of the endothelium and respective basement membrane with their end-feet, also known as the glia limitans perivascularis. Altogether, these densely packed cells and structure as whole maintains and stabilizes the BBB Fig. (1). In general, a CNS-penetrating drug has to be hydrophobic or small enough to cross this impressive fortification effectively. However, most newly-discovered neuroprotective substances are hydrophilic peptides or proteins that cannot cross the BBB on their own.

In addition to its restrictive physical properties, the BBB has an active enzymatic system to curtail the effective half-life of perceived toxins and to remove infiltrating foreign substances Fig. (1). For example, the enkephalin metabolizing enzymes, aminopeptidase M, angiotensin converting enzymes and neutral endopeptidase are all active enzymatic barriers to prevent the free passage of many peptides through the BBB [4].

Finally, the BBB is also reinforced by a group of active efflux transport proteins Fig. (1), including P-glycoprotein (P-gp), multidrug resistance protein (MRP), and breast cancer resistance protein (BCRP), all of which belong to the ATP-binding cassette protein family [5]. A high concentration of P-gp is expressed in the luminal membranes of the cerebral capillary endothelium. It is the most important efflux system and actively removes many compounds from the endothelial cell cytoplasm before they penetrate the brain parenchyma. BCRP and several members in the MRP family (MRP-1, MRP3 and MRP5) are also expressed in the BBB where they help to maintain brain homeostasis and remove toxic compounds.
To promote the passage of therapeutic agents into the CNS, a variety of approaches have been attempted, including disruption of the BBB [6], bypassing the BBB [7], blocking efflux transporters [8], and using drug carriers such as cell-penetrating peptides (CPPs) [9], antibodies or ligands based on receptors on the BBB [10], liposomes [11], and nanoparticles (NPs) [12]. The current review will focus on non-invasive drug carriers, with a special emphasis on the CPPs.

**CELL-PENETRATING PEPTIDES DERIVED FROM PROTEIN TRANSDUCTION DOMAINS FOR CNS DRUG DELIVERY**

Analysis of naturally occurring proteins that are able to circumvent membranous barriers has led to the discovery of several protein sequences known as protein transduction domains (PTD). CPPs are small peptides derived from PTD that retain the cell-membrane-permeable properties of the native proteins. When attached to the molecule of interest via either chemical or genetic means, CPPs confer their cargo with the ability to cross cellular membranes and gain access to CNS targets.

CPPs are derived from various sources (Table 1). Naturally derived CPPs, including the TAT peptide from Human Immunodeficiency Virus (HIV) transcription-activating factor [13] and penetratin peptide from the Drosophila antennapedia protein [14] are prototypical CPPs and have been used extensively. Synthetic CPPs, such as homoarginine peptides and transportan, are designed with predetermined structures and are easily modified with different chemical entities, which give rise to novel and more efficient therapeutic agents [15, 16]. Chimeric CPPs, such as the sequence signal-based peptide, combine sequences with different functions to achieve synergistic translocation efficiency [17].

Although heterogeneous in size and sequence, CPPs share a few common properties. (1) All CPPs are small peptides between 6 to 30 amino acids long. (2) Nearly all CPPs are positively net charged at physiological pH due to the high content of basic amino acids (e.g., arginine and lysine) in their protein sequences. This positive charge seems to be critical for the cellular uptake of CPPs. The substitution of basic residues in CPPs with neutral alanines reduces cell penetration, while substitution of neutral residues with arginines actually enhances internalization [18, 19]. The cationic nature itself, however, is not sufficient for optimal translocation of CPPs. Other features, such as the number of arginine residues are also important for their translocation activity [20]. (3) A vast majority of CPPs are characterized by an amphipathic structure while showing overall hydrophobicity. Of course, a certain degree of hydrophobicity can help the cationic peptide sequences enter target cells, but a highly hydrophobic peptide would be trapped in the membrane rather than being internalized [21]. In short, an amphipathic structure is critical for the internalization of CPPs.

In addition to its BBB permeability, several other advantages of the CPP delivery system have made it one of the most widely exploited drug carriers in CNS studies. First, CPP-mediated delivery is surprisingly independent of cargo size. Molecules range in size from small peptides, proteins, oligonucleotides, imaging agents to massive structures such as nanoparticles and liposomes have all been successfully delivered into the brain using CPPs. Second, the
<table>
<thead>
<tr>
<th>CPP</th>
<th>Sequence</th>
<th>Source</th>
<th>Refs</th>
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</thead>
<tbody>
<tr>
<td>Naturally-derived</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>TAT</td>
<td>GRKKRQRRRPPQ</td>
<td>HIV transcription-activating factor</td>
<td>[22]</td>
</tr>
<tr>
<td>Penetratin</td>
<td>RQIKIWFQNRRMKWKK</td>
<td>Drosophila homeoprotein antennapedia</td>
<td>[23]</td>
</tr>
<tr>
<td>VP22</td>
<td>DAATATGRSAASRPTERRRPRPVD</td>
<td>HSV VP22 protein</td>
<td>[24]</td>
</tr>
<tr>
<td>SynB1</td>
<td>RGGRLSYSRRRFSTSTGR</td>
<td>Protegrin</td>
<td>[25]</td>
</tr>
<tr>
<td>pVEC</td>
<td>LLIILRRRIRKQAHASHK</td>
<td>Murine vascular endothelial cadherin</td>
<td>[26]</td>
</tr>
<tr>
<td>SAP</td>
<td>(VRLPPP)</td>
<td>Modified maize γ-zein-related sequence</td>
<td>[27]</td>
</tr>
<tr>
<td>hCT</td>
<td>LGTYQTDFNKFHTFPQTAIGVGAP</td>
<td>Human calcitonin</td>
<td>[28]</td>
</tr>
<tr>
<td>BagP</td>
<td>MKKKTRRRRT</td>
<td>Human protein bag-1</td>
<td>[29]</td>
</tr>
<tr>
<td>HP4</td>
<td>RRRRPRRTTRRRRR</td>
<td>Herring portamine</td>
<td>[19]</td>
</tr>
<tr>
<td>Lactoferrin (hLF)</td>
<td>KCFQWQRNMRKVRGPPVSCIKR</td>
<td>Human milk protein lactoferrin</td>
<td>[30]</td>
</tr>
<tr>
<td>Nucleolar targeting peptides</td>
<td></td>
<td>Crotamine, a toxin in venom of the South American rattlesnake</td>
<td>[31]</td>
</tr>
<tr>
<td>Cytoplasmic Localizing Peptide-1 (CyLoP-1)</td>
<td>CRWRWKCCKK</td>
<td>Crotamine, a toxin in venom of the South American rattlesnake</td>
<td>[32]</td>
</tr>
<tr>
<td>gH625</td>
<td>HGLASTLTRWAHYNALIRAGGG</td>
<td>glycoprotein gH of herpes simplex type I virus</td>
<td>[33]</td>
</tr>
<tr>
<td>Synthesized</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model amphipathic peptide (MAP),</td>
<td>KLALKLALKALAALKLA</td>
<td>Synthetic</td>
<td>[34]</td>
</tr>
<tr>
<td>Homoarginine peptides</td>
<td>RRRRRRR(RR)</td>
<td>Synthetic</td>
<td>[20]</td>
</tr>
<tr>
<td>C105Y</td>
<td>CSIPVEVKFNKPFVYL</td>
<td>Synthesized based on the amino acid sequence corresponding to residues 359-374 of alpha1-antitrypsin</td>
<td>[35]</td>
</tr>
<tr>
<td>M918</td>
<td>MVTVLFRRRLIRRACPVRV</td>
<td>Synthetic</td>
<td>[36]</td>
</tr>
<tr>
<td>S41</td>
<td>CVQWSLLRGYQPC</td>
<td>Synthetic</td>
<td>[37]</td>
</tr>
<tr>
<td>CACY</td>
<td>GLWRALWRLLRSLWLLWRA</td>
<td>Synthetic</td>
<td>[38]</td>
</tr>
<tr>
<td>Chimeric</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPG</td>
<td>GALFLGFLGAAGSTM-GAWSQPKKRRKV</td>
<td>A hydrophobic domain from the fusion sequence of HIV gp41 and a hydrophilic domain from the NLS of SV40 T-antigen</td>
<td>[39]</td>
</tr>
<tr>
<td>Signal sequence-based peptide</td>
<td>MGLGLHLIVLAAALQ-GAWSQPKKRRKV</td>
<td>A hydrophobic domain from Caiman crocodylus Ig(v) light chain and a hydrophilic domain from the NLS of SV40 large T antigen</td>
<td>[17]</td>
</tr>
<tr>
<td>Transportan 10</td>
<td>GWTLNSAGYLLGKINLKAALAKKIL</td>
<td>12 amino acids from the N-terminal of neuropeptide galanin and 14 amino acid long wasp venom peptide, mastoparan, connected via a lysine residue</td>
<td>[40]</td>
</tr>
<tr>
<td>PEP-1</td>
<td>KETWWETWWTEWSQPKKRRKV</td>
<td>a hydrophobic tryptophan-rich motif and a hydrophilic lysine-rich domain derived from the nuclear localization sequence (NLS) of simian virus 40 (SV-40) large T antigen, separated by a spacer domain (SQP)</td>
<td>[41]</td>
</tr>
<tr>
<td>S4(13)-PV</td>
<td>ALWKTLKKVLKAPKKRRKV</td>
<td>a 13 amino acid cell-penetrating sequence derived from the dermaseptin S4 peptide and the NLS of SV40 large T antigen</td>
<td>[42]</td>
</tr>
</tbody>
</table>
in vivo systemic delivery of CPPs lead to rapid expression of cargo molecules in the CNS cells. For example, certain proteins constructed using TAT-PTD technology can cross the BBB and transduce into CNS cells in a matter of mere hours after systemic administration [9, 43]. Finally, peptide-mediated delivery of macromolecules is superior to other commonly used delivery systems in many aspects, such as relatively low toxicity, high delivery efficiency, and the possibility for peptide backbone modifications. Hence, the use of CPPs opened a new gateway for CNS drug delivery and is a promising approach for treating neurological diseases. In this section, select CPPs used frequently for BBB penetration and their application in CNS diseases will be discussed.

HIV TRANSCRIPTION-ACTIVATING FACTOR TAT PEPTIDE

The transactivator of transcription protein of the HIV is an 86 amino acid protein that plays an essential role in virus replication. In 1988, two research groups independently discovered that the HIV TAT protein can penetrate cells efficiently when added exogenously in tissue culture [13, 44]. It was also found that the transduction property could be attributed to the amino acid counterpart of the gene sequence position 37-72 [44]. A subsequent study [22] more precisely located the 11-amino acid sequence (YGRKKRR QRRR) at amino acids 47–57, now known as the TAT peptide as the essential CPP for TAT protein.

It was then demonstrated in 1994 that several large proteins, when conjugated to TAT transduction peptides at amino acids 1-72 or 37-72, could enter various types of cells in culture [45]. For example, TAT-conjugated β-galactosidase was shown to distribute to cells of multiple organs, but not the brain, after systemic delivery [46]. Later on, by constructing fusion proteins with the essential TAT-PTD at their amino- or carboxy-terminus, an improved TAT delivery system was shown to mediate an efficient and rapid expression of fusion protein in various tissues after intraperitoneal injection [9]. Spectacularly, the cargo protein activity was detected in brain neurons without impairment of the BBB, as manifested by the lack of Evans blue dye brain infiltration [9]. This attraction of TAT-PTD for the brain and non-dividing neurons has particularly important implications in neuroscience research and in CNS drug design. In recent years, different modifications of TAT-CPP have successfully enhanced its efficient transduction into cultured cells and increase its bioavailability and activity in vivo [47-49]. The application of the TAT-based delivery system has extended from peptides/proteins to a wide variety of cargoes (Table 2). In the later section, we will discuss in detail the application of TAT in stroke.

PEP-1

Pep-1 is another CPP frequently used in CNS studies. It is a chimeric CPP with an amphipathic sequence. It contains a hydrophobic tryptophan-rich motif (KETWWETWWTEW), which is responsible for the interaction with proteins and cell membranes, a hydrophilic lysine-rich domain derived from the nuclear localization sequence (NLS) of simian virus 40 (SV-40) large T antigen (KKKKKK), which improves solubility, and a spacer domain in between (SQP), which improves the flexibility and the integrity of the other two domains [41]. When mixed with target proteins (GFP, β-gal), Pep-1 peptide led the nondenatured target protein into cultured cells.

A number of studies have demonstrated that Pep-1 is able to deliver a variety of proteins and peptides into the brain. A fusion protein of antioxidant enzyme Cu,Zn-superoxide dismutase (SOD) and Pep-1 can cross the BBB after intraperitoneal injection and transduce into neurons [50]. A series of studies reported that this Pep-1-SOD fusion protein protected against transient forebrain ischemia [50-52] and spinal cord injury [53, 54]. Pep-1 also successfully delivered other neuroprotective proteins into the brain, including copper chaperone for SOD (CCS) [55], ribosomal protein S3 (rPS3) [51], HSP27 [56], catalase [57] and Frataxin [58] to reduce post-ischemic injury.

PENETRATIN

Penetratin is derived from Drosophila homeoprotein Antennapedia (Antp) and belongs to a class of trans-activating factors that are critical in several morphological processes including neuronal morphogenesis [14, 59]. These homeoproteins bind DNA through a specific sequence of 60 amino acids called the homeodomain. It was found that the homeodomain of Antp has the capacity to translocate across the neuronal membrane [14]. Structurally, the homeodomain consists of three α-helices with one β turn between helix 2 and 3 [60]. Subsequent studies identified a 16-amino-acid sequence (KETWWETWWTEW), so-called penetratin, in the third helix of the Antp homeodomain that is involved in the translocation process and is the minimal CPP of Antp [23].

The ability of penetratin to deliver cargoes across the BBB was first shown by Roussel and colleagues. In their study, penetratin CPP enabled a covalently coupled chemotherapeutic agent to cross the cellular membranes of the BBB with high efficiency without compromising BBB integrity [25]. It has also been shown that penetratin can help small proteins, such as C-terminal structure BIR3-RING of X-linked inhibitor of apoptosis protein (XIAP) [61] and calbindin D [62], enter rat brains after systemic application and thereby decrease transient middle cerebral artery occlusion (MCAO) induced cell apoptosis. Penetratin was also conjugated with polyethyleneimine (PEI) and polyamidoamine (PAMAM) to prepare Antp-modified DNA-loaded nanoparticles, which enhances the transfection and expression efficiency of DNA/PAMAM nanoparticle in brain capillary endothelial cells [63].

SYNB VECTORS

The SynB peptide family is derived from a natural antimicrobial peptide called protegrin 1 (PG1) [64]. PG1 is 18 amino acids long (RGGRLCYSRRFCCVCG), forming an antiparallel β-sheet structure constrained by two disulfide bridges [65]. It interacts with and disrupts the lipid matrix of bacterial membranes [66]. SynB1 is a linear analogue of PG1 with the four cysteine residues replaced by serines and two valines replaced by threonines [25]. SynB1 is able to cross cell membranes efficiently and is devoid of any cytolytic effect of PG-1. Further optimizations have led to the development of shorter SynB peptides with improved translocation properties [67].

Several drugs conjugated to SynB vectors have demonstrated increased brain uptake and in vivo activities [25, 68, 69]. For example, the BBB non-permeable doxorubicin can gain access to the brain after systemic application when it is covalently linked to SynB1 peptide [25]. Interestingly, this delivery strategy may reduce the cardiotoxicity of doxorubicin, as the heart had significantly lower SynB1-doxorubicin levels. The SynB vectors have also been used to deliver peptide molecules such as enkephalin and streptavidin to the brain [70]. A recent study designed a novel delivery system that contains SynB1 and a thermally responsive elastin-like polypeptide (ELP). It was shown that this SynB-ELP polypeptide could enhance thermal-based therapeutics targeting to various regions of the brain, particularly the cerebellum [71].

OTHER CPPS FOR CNS DELIVERY

Several other CPPs can penetrate the BBB after systemic application. For example, a synthetic arginine-rich CPP ([KRRBR]XB) facilitated the brain uptake of antisense morpholinolo oligonucleotides [72], which can induce alternative pre-mRNA splicing and thereby offer a potential therapeutic tool for neurogenetic disorders such as ataxia-telangiectasia [73]. In another study, a basic amino acid-rich nucleolar localization signal in LIM kinase 2 (LIMK2 NoLS) showed potential to transport peptides and proteins across
### Table 2. Cargoes Delivered Across Blood Brain Barrier by TAT Cell Penetrating Peptides Delivery System in Stroke and other CNS Disease Models

<table>
<thead>
<tr>
<th>Cargo</th>
<th>Formula</th>
<th>Route</th>
<th>Animal Model</th>
<th>Biological Effect</th>
<th>Refs</th>
</tr>
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<tbody>
<tr>
<td>Peptide and Protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bcl-x(L)</td>
<td>TAT-Bcl-x(L) fusion protein</td>
<td>iv or ip</td>
<td>Mouse, Ischemia reperfusion (I/R)</td>
<td>Anti-apoptotic; attenuates ischemia-induced brain injury; increases survival of neural precursor cells</td>
<td>[43, 97, 102, 103]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ip</td>
<td>Rat, neonatal HI</td>
<td>Anti-apoptotic; decreases cerebral tissue loss</td>
<td>[126]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ip</td>
<td>Gerbils, transient forebrain ischemia</td>
<td>Prevents delayed neuronal death in the hippocampus</td>
<td>[102]</td>
</tr>
<tr>
<td>X chromosome-linked inhibitor of apoptosis (XIAP)</td>
<td>TAT-XIAP fusion protein</td>
<td>iv</td>
<td>Mouse, permanent ischemia</td>
<td>Anti-apoptotic; efficiently targets the lesion, reduces infarct volumes, reverses long-term impairments</td>
<td>[100]</td>
</tr>
<tr>
<td>Glial-cell-line-derived neurotrophic factor (GDNF)</td>
<td>TAT-GDNF fusion protein</td>
<td>iv</td>
<td>Mouse, I/R</td>
<td>Neurotrophic factor; prevents neuronal death and reduces infarct volume</td>
<td>[99]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ip</td>
<td>Mouse, MPTP model of Parkinson’s disease</td>
<td>Neurotrophic factor; reaches dopaminergic neurons but elicits no protection</td>
<td>[127]</td>
</tr>
<tr>
<td>Erythropoietin (EPO)</td>
<td>TAT-EPO fusion protein</td>
<td>ip or iv</td>
<td>Mouse, I/R</td>
<td>TAT enhances the capacity of EPO to cross the BBB at least twofold when given IP and up to five-fold when given IV, TAT-EPO protects against ischemic brain injury.</td>
<td>[108]</td>
</tr>
<tr>
<td>Inhibitor of δPKC (δV1-1)</td>
<td>δV1-1-TAT fusion protein</td>
<td>ip</td>
<td>Rat, I/R</td>
<td>Improves microvascular pathology, increases cerebral blood flow, reduces infarct size</td>
<td>[101]</td>
</tr>
<tr>
<td>ψε-Receptors for Activated C Kinase (RACK)</td>
<td>TAT-ψε-RACK fusion protein</td>
<td>iv</td>
<td>Rat, Global cerebral ischemia</td>
<td>Activation of εPKC by TAT-ψε-RACK protects the brain against ischemic damage by regulating cerebral blood flow</td>
<td>[114]</td>
</tr>
<tr>
<td>c-Jun N-terminal kinase-inhibitor (D-JNKI1)</td>
<td>TAT-DJNKI1 fusion protein</td>
<td>ip</td>
<td>Rat/mouse, permanent ischemia</td>
<td>JNK inhibitor; reduces lesion volume and improves neurological function</td>
<td>[115-117]</td>
</tr>
<tr>
<td>JNK-inhibitor JBD</td>
<td>TAT-JBD fusion protein</td>
<td>ip</td>
<td>Rat, neonatal HI</td>
<td>Reduces neonatal HI brain damage with long-lasting anatomical and behavioral improvements</td>
<td>[128]</td>
</tr>
<tr>
<td>Postsynaptic density-95 (PSD95) inhibitor</td>
<td>Tat-NR2B9c fusion protein</td>
<td>iv</td>
<td>Rat, I/R and permanent ischemia</td>
<td>Blocks NMDAR-PSD95 signaling, reduces infarct volumes and improves long-term neurobehavioral functions</td>
<td>[119, 120]</td>
</tr>
<tr>
<td>Neuroglobin</td>
<td>TAT-Neuroglobin fusion protein</td>
<td>iv</td>
<td>Mouse, I/R</td>
<td>Efficiently transduces into neurons and protects the brain from ischemia</td>
<td>[97]</td>
</tr>
<tr>
<td>Nogo-A extracellular peptide residues 1-40 (NEP1-40)</td>
<td>TAT-NEP1-40 fusion protein</td>
<td>ip</td>
<td>Mouse/rat, I/R</td>
<td>Inhibits neuronal death, promotes axonal regrowth and functional recovery</td>
<td>[122, 123]</td>
</tr>
<tr>
<td>Hsp70</td>
<td>TAT-Hsp70 fusion protein</td>
<td>iv</td>
<td>Mouse, I/R</td>
<td>Chaperone; reduces infarct volume and increases survival of neural precursor cells</td>
<td>[124]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ip</td>
<td>Mouse, MPTP model of Parkinson’s disease</td>
<td>Chaperone; prevents neuronal cell death</td>
<td>[130]</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>TAT-SOD fusion protein</td>
<td>ip</td>
<td>Rat, I/R</td>
<td>Increases SOD activity in the brain and protects the brain from I/R injury</td>
<td>[125]</td>
</tr>
<tr>
<td>Cargo</td>
<td>Formula</td>
<td>Route</td>
<td>Animal Model</td>
<td>Biological Effect</td>
<td>Refs</td>
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<tr>
<td>Apaf-1-inhibitory protein (AIP)</td>
<td>TAT-AIP fusion protein</td>
<td>ip</td>
<td>Gerbils, transient forebrain ischemia</td>
<td>Prevents neuronal death in hippocampus</td>
<td>[131]</td>
</tr>
<tr>
<td>NF-κB essential modulator (NEMO) binding domain (NBD)</td>
<td>TAT-NBD fusion protein</td>
<td>ip</td>
<td>Rat, neonatal hypoxia-ischemia (HI)</td>
<td>Anti-apoptotic; decreases brain damage and improves neurological functions</td>
<td>[132]</td>
</tr>
<tr>
<td>Sensitive to apoptosis gene (SAG)</td>
<td>TAT-SAG fusion protein</td>
<td>ip</td>
<td>Gerbils, transient forebrain ischemia</td>
<td>Prevents neuronal death in hippocampus, decreases lipid peroxidation in the brain</td>
<td>[135]</td>
</tr>
<tr>
<td>Leptin</td>
<td>TAT-leptin</td>
<td>ip</td>
<td>Mouse, fed with high-fat diet</td>
<td>Increases leptin in hippocampus, reduces body weight</td>
<td>[136]</td>
</tr>
<tr>
<td>Lipoamide dehydrogenase (LAD, E3)</td>
<td>TAT-LAD fusion protein</td>
<td>iv</td>
<td>E3-deficient mice</td>
<td>Causes significant increase in the enzymatic activity of the mitochondrial multienzyme complex pyruvate dehydrogenase complex within the liver, heart and brain</td>
<td>[137]</td>
</tr>
<tr>
<td>Ubiquitin c-terminal hydrolase L1 (Uch-L1)</td>
<td>TAT-HA-Uch-L1 fusion protein</td>
<td>s.c</td>
<td>Mouse, APP/PSI Alzheimer model</td>
<td>Restores spine density</td>
<td>[138]</td>
</tr>
<tr>
<td>Ciliary neurotrophic factor (CNTF)</td>
<td>TAT-CNTF</td>
<td>Ipvper</td>
<td>Mouse, Aβ-induced dementia</td>
<td>Neurotrophic factor, reduces learning and memory impairments</td>
<td>[139]</td>
</tr>
<tr>
<td>Choline acetyltransferase</td>
<td>TAT-choline acetyltransferase fusion protein</td>
<td>iv</td>
<td>Mouse, Aβ-induced dementia</td>
<td>Neurotransmitter, improves memory and cognitive dysfunction</td>
<td>[140]</td>
</tr>
<tr>
<td>Imaging agents</td>
<td></td>
<td></td>
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<tr>
<td>Cy5.5</td>
<td>Cy5.5-TAT</td>
<td>iv</td>
<td>Rat, experimental autoimmune encephalomyelitis</td>
<td>Labels and tracks T lymphocyte</td>
<td>[141]</td>
</tr>
<tr>
<td>CdS ratio Mn/ZnS quantum dots (Qdots),</td>
<td>TAT-Qdots</td>
<td>Intra-artery</td>
<td>Rat</td>
<td>Reaches the brain parenchyma for imaging</td>
<td>[142]</td>
</tr>
<tr>
<td>FITC doped silica nanoparticles</td>
<td>TAT-FSNPs</td>
<td>Intra-artery</td>
<td>rat</td>
<td>Used for brain imaging</td>
<td>[143]</td>
</tr>
<tr>
<td>Liposomes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doxorubicin-loaded liposome</td>
<td>TAT conjugated with cholesterol for doxorubicin-loaded liposome</td>
<td>iv</td>
<td>Rat, Brain glioma</td>
<td>Increases survival time of glioma-bearing rats; distribution of doxorubicin increases in the brain and decreases in heart</td>
<td>[144]</td>
</tr>
<tr>
<td>Nanoparticles</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Coumarin-loaded methoxy poly(ethylene glycol) (MPEG)/poly(e-caprolactone) (PCL)</td>
<td>Coumarin-loaded MPEG-PCL - TAT</td>
<td>Intra-nasal</td>
<td>Rat</td>
<td>Nano-sized micelles modified with Tat facilitate direct intranasal brain delivery</td>
<td>[145]</td>
</tr>
</tbody>
</table>
the BBB [74]. With rapid advancements in technology, an increasing number of CPPs will be discovered, designed, or modified to enable brain delivery of therapeutic biomolecules.

**RECEPTOR-MEDIATED SPECIFIC TRANSCYTOSIS**

Receptor-mediated specific transcytosis is a delivery method based on the physiological receptors expressed on endothelial cells forming the BBB. The molecule of interest is conjugated with the monoclonal antibodies or ligands to these physiological receptors, which lead to the translocation of fusion products through the BBB. So far, several receptors, including the insulin receptor and transferrin receptor, have been exploited for delivering compounds across the BBB in experimental stroke studies.

**TRANSFERRIN RECEPTOR**

Serum transferrin functions as a transporter carrying iron from the site of intake to various tissues and cells. Transferrin draws wide attention in the drug delivery area due to its nonimmunogenicity, low toxicity, as well as biodegradability [75]. In addition, the high expression levels of transferrin receptors (TfR) on CNS endothelial cells relative to other organs makes this vector an ideal candidate for CNS-targeted delivery [75]. One disadvantage, however, is that the transferrin vector applied in vivo has to compete with large amounts of endogenous transferrin for BBB TfR binding sites, which significantly diminishes its delivery efficiency [76]. To overcome this problem, a monoclonal antibody against the TfR named OX26 was generated. This antibody binds to a distinct site on TfR from the binding site for endogenous transferrin and thus cannot be outcompeted. Using this approach, nerve growth factor (NGF) was found to penetrate the BBB in a dose-dependent fashion following intravenous injection and prevented the degeneration of cholinergic striatal neurons in a rat model of Huntington’s disease [10]. In another study, a single intravenous injection of BDNF-OX26 protected neurons in rats subjected to 60-min MCAO [77]. In order to further strengthen the binding between the drug and OX26 as well as to reduce the drug modification, the high affinity of streptavidin for the biotin molecule was exploited in this system. Briefly, a conjugate of OX26 and streptavidin was produced in parallel with the monobiotinylated drug [78]. The subsequent conjugate of biotinylated FGF and OX26-streptavidin was shown to be effective in reducing brain infarct volume and in improving neurological deficits in rats subjected to permanent MCAO [79].

**INSULIN RECEPTOR**

Small amounts of circulating insulin can cross the BBB into the CNS through insulin receptors on endothelial cells. The monoclonal antibody to human insulin receptor (HIRMAb) thus acts as a Trojan horse to ferry a molecule of interest across the BBB when this molecule is fused to the carboxyl terminus of the heavy chain of HIRMAb. Exemplary HIRMAb fusion proteins used for the uptake of various drugs into the brain are listed in (Table 3). Among these fusion proteins, HIRMAb-EPO and HIRMAb-GDNF have been shown to cross the BBB after intravenous injection in rhesus monkeys [80] and both protect against cerebral ischemia following intracerebral administration in rat model of stroke [81, 82].

Although many studies observed an increase in brain uptake of the receptor ligand/antibody-conjugated proteins from the periphery compared to the corresponding unmodified proteins and reported the effectiveness of these conjugated proteins in models of neurological diseases after systemic application, several issues remain poorly addressed. For instance, it is unclear if and how fusion with antibodies or ligands alters the activity of the modified protein. In addition, given that all these targeted BBB receptors are also expressed on peripheral organs and play important physiological roles, this raises the concern that the effective dosage to enter brain parenchyma may lead to unbearable peripheral side effects. Furthermore, there is also the possibility that chronic repeated injection of the fusion protein in vivo may cause the down-regulation of targeted receptors during the therapeutic period.

**NANOPARTICLE-MEDIATED DELIVERY**

Recently, various types of nanoparticles (NPs) infused with therapeutic agents have been developed as efficient, site-specific and control-released modes of drug delivery. NPs used for drug delivery consist of different materials such as polymers, lipids or metals. In principle, the biodegradable and stable polymers such as poly (alkyl cyanoacrylate) (PBCA), poly (lactide-co-glycolide) (PLGA), human serum albumin [83] are excellent candidates. Drugs can either be integrated into the matrix of the particle or attached to the particle surface. These NPs allow peptide cargo to gain access to the surface of the endothelial cells forming the BBB and to penetrate cell membranes. Coating the NPs with certain surfactants such as polysorbate 80 (PS 80) [84] or attaching the NPs with certain ligands such as transferrin [85] or lipoprotein [86] could redistribute the NPs and enhance their accumulation in the cerebral parenchyma. These pre-coating modified NPs also reduce kidney and liver toxicity because of their targeted distribution [87]. This system has already been effective against in vivo models of ischemic stroke [88], brain tumors [87, 89], Alzheimer’s disease [90], and Parkinson’s disease [91].

The internalization of NPs depends on the cell type, the size of the particles, the surface charge, and the hydrophobicity of the particles [92]. For instance, small particles below 200 nm are probably internalized through clathrin-mediated endocytosis, while the uptake of larger particles up to 500 nm may be caveolae-mediated.

<table>
<thead>
<tr>
<th>Cargo</th>
<th>Formula</th>
<th>Route</th>
<th>Animal Model</th>
<th>Biological Effect</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol-conjugated antimicrobial peptides G3R(6)</td>
<td>Cholesterol-conjugated G3R(6)-TAT</td>
<td>iv</td>
<td>Rabbit. C.neoformans meningitis model</td>
<td>Antimicrobial; Crosses the BBB and suppresses yeast growth in brain tissues</td>
<td>[146]</td>
</tr>
<tr>
<td>Ciprofloxacin-loaded cholesterol-conjugated poly(ethylene glycol) (PEG)</td>
<td>TAT-PEG-b-Col-Ciprofloxacin</td>
<td>iv</td>
<td>Rat</td>
<td>Antimicrobial; crosses the BBB and enters the brain</td>
<td>[147, 148]</td>
</tr>
<tr>
<td>Ritonavir-loaded Poly(L-lactide) (PLA) nanoparticle,</td>
<td>Ritonavir-PLA-TAT</td>
<td>iv</td>
<td>Mouse</td>
<td>Enhances the CNS bioavailability of the encapsulated PI and maintains therapeutic drug levels in the brain for a sustained period</td>
<td>[149]</td>
</tr>
</tbody>
</table>
has also been reported that PS 80 coated PBCA NPs have the ability to adsorb apolipoprotein E and B in the plasma after intravenous injection and therefore cross the BBB through low-density lipoprotein receptor-related protein (LRP) [93].

**IMMUNE CELL-BASED DELIVERY**

Recent research suggests that immune cells might also be feasible drug delivery vectors [94, 95]. This approach exploits the natural ability of peripheral immune cells such as macrophages to pass the BBB and home to sites of neuronal injury or degeneration to endocytose cellular debris. For example, transplantation of genetically modified macrophages overexpressing GDNF protects against MPTP-induced dopaminergic neurodegeneration [94]. Another study demonstrated that macrophages could deliver the vectors of NPs into gliomas for photothermal ablation [95]. Finally, it is well known that the infiltration of peripheral immune cells into the ischemic core and penumbra occurs immediately after stroke and remains prominent for several days [96]. In this regard, immune cell-based delivery is a highly promising approach to rapidly deliver neurotherapeutics to the ischemic brain for an early rescue of injured neurons. A distinct advantage of this approach is its cellular specificity, as the immune cells are not likely to target uninjured sites, either in the brain or the periphery.

**TAT-MEDIATED CNS DELIVERY OF THERAPEUTIC PEPTIDES AND PROTEINS IN PRE-CLINICAL STROKE RESEARCH**

Despite our growing understanding of the critical mechanistic events in post-stroke brain injury, there has been little clinical translation of these findings. One important limiting factor is the inability of systemically applied strategies to efficiently cross the BBB and enter brain cells. Several viral vectors, especially the adeno-associated virus (AAV), have shown great promise to achieve robust transgene expression in the brain. However, given the relatively acute nature of cell death after stroke and the small time window of opportunity for stroke treatment, gene delivery, which necessitates a relatively long time for protein expression, is not a feasible option. Additionally, the expression of viral vector-delivered genes relies on host machinery for protein synthesis, which is often severely compromised during ischemic injury. Thus far, the application of viral vectors is therefore mostly in the research arena.

An alternative approach for CNS delivery of neurotherapeutics after stroke is to directly send the gene product itself into the brain. CPP is a commonly used strategy under this category. As a nonviral system, the in vivo delivery of CPP does not require a prolonged incubation period to achieve gene expression. This advantage allows for its therapeutic application in acute CNS diseases such as stroke. According to most studies, TAT coupled proteins are detected in the brain as early as 4 hours after systemic injection [43, 97-99], a timeframe that is within the therapeutic window for stroke. More intriguingly, some of these proteins have proven protective even when applied after ischemic onset [43, 98-101]. All of these advantages encourage the translation of these treatments into clinical use. Quite a few peptides and proteins have been successfully delivered into the brain by CPP for the treatment of stroke and other neurological disorders (Table 2). In this section, we will use TAT as a prototypical CPP and review several specific TAT fusion proteins that cross the BBB and exert neuroprotection against cerebral ischemia.

**ANTI-APOPTOTIC MOLECULES**

In 2002, three independent research groups [43, 83, 98] reported that systemically administered TAT-Bcl-xL penetrated the brain and protected neurons against focal ischemia/reperfusion injury. Our study [43] showed that intraperitoneally applied TAT-HA-Bcl-xL could be detected in brain tissue by Western blot at 4 hours after injection. Dose-dependent increases in the amount of TAT-HA-Bcl-xL were detected by either anti-HA or anti-Bcl-xL antibody in various brain regions including the cortex, caudoputamen, hippocampus, cerebellum and spinal cord. In contrast, the HA-Bcl-xL without TAT could not achieve detectable protein transduction. Quantitative ELISA confirmed that transduced Bcl-xL reached concentrations of 4 ng/mg in cortex tissue. Importantly, this TAT-HA-Bcl-xL fusion protein was protective against 90-min transient focal ischemia even when applied 45 min after ischemia onset. Similarly, Kilic and colleagues showed that intravenously injected TAT-Bcl-xL greatly reduced infarct size and improved neurological performance after 90-min of MCAO [98]. TAT-Bcl-xL also exerted neuroprotec-

**Table 3. Insulin Receptor-Mediated CNS Delivery**

<table>
<thead>
<tr>
<th>Fusion protein</th>
<th>Route</th>
<th>Animal Model</th>
<th>Functions</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIRMAb-EPO fusion protein (Erythropoietin conjugated to human insulin receptor monoclonal antibody)</td>
<td>iv</td>
<td>rhesus monkeys</td>
<td>HIRMAb-EPO fusion protein is rapidly transported into brain</td>
<td>[80]</td>
</tr>
<tr>
<td>HIRMAb-EPO fusion protein</td>
<td>icv</td>
<td>rat permanent MCAO</td>
<td>Reduces infarct volume and reduces neurological deficits</td>
<td>[81]</td>
</tr>
<tr>
<td>HIRMAb-GDNF (Gial-derived neurotrophic factor bind to HIRMAb)</td>
<td>icv</td>
<td>rat permanent MCAO</td>
<td>The fusion protein is active in vivo and reduces infarct volume</td>
<td>[82]</td>
</tr>
<tr>
<td>HIRMAb-IDS fusion protein (iduronate-2-sulfatase conjugated to HIRMAb)</td>
<td>iv</td>
<td>Mucopolysaccharidosis MCAO [150] Type II</td>
<td>Produces therapeutic concentrations of IDS in the brain</td>
<td>[151]</td>
</tr>
<tr>
<td>HIRMAb-TNFR fusion protein (type II TNF receptor conjugated to HIRMAb)</td>
<td>iv</td>
<td>rhesus monkeys</td>
<td>Fusion protein selectively targets brain more than peripheral organs; 3% of the injected dose is taken up by the primate brain</td>
<td>[80]</td>
</tr>
<tr>
<td>HIRMAb-ScFv (a single chain Fv antibody against the Abeta peptide conjugated to HIRMAb)</td>
<td>iv</td>
<td>rhesus monkeys</td>
<td>Can be transported across the primate BBB in vivo</td>
<td>[152]</td>
</tr>
</tbody>
</table>
tion against a 30-min or a 2-hour focal ischemia. In another study, a derivative of Bcl-xL with a more flexible and mobile pore-forming domain was generated and designated as FNK [83]. FNK has a higher protective activity than wild-type Bcl-xL. The TAT-myc-FNK fusion protein accessed brain neurons after intraperitoneal injection and prevented delayed neuronal death in the hippocampus caused by 5-min global ischemia. Subsequent studies using TAT-Bcl-xL fusion protein demonstrated that this protein not only inhibits ischemia-induced cell death, but also enhances the survival of neural precursor cells and improves hippocampal neurogenesis after transient ischemia [102, 103].

X chromosome-linked inhibitor of apoptosis (XIAP), a potent inhibitor of both caspase-3 and apoptosis, is a 62-kDa protein that does not cross BBB by itself. TAT fused XIAP, however, could transduce cortical cells even when applied topically. Shorter constructs could successfully target brain lesions after intravenous delivery and significantly reduced infarct volumes induced by distal occlusion of the MCA [100].

TROPHIC FACTORS

Many trophic factors have shown high potential for neuroprotective applications. However, they usually do not readily cross the BBB. For example, glial cell line-derived neurotrophic factor (GDNF) is a potent neurotrophic factor that promotes neuronal survival [104]. Kilic and colleagues showed robust GDNF transduction in the brain of TAT-GDNF-treated animals 4 hours after intravenous application [99]. TAT-GDNF administered either before or 30 minutes after onset of ischemia was neuroprotective in a MCAO model of ischemic stroke, suggesting that this protein may be a powerful tool for the immediate treatment of stroke.

It is now widely known that erythropoietin [41] is not only a haematopoietic factor, but also a multifunctional trophic factor with neuroprotective effects in animal models of ischemia [105] and in stroke patients [106]. However, the BBB permeability of EPO is very low as only 0.5-1% of systemically administered EPO crosses the BBB [107]. To reach therapeutic concentrations in the brain, either large amounts or multiple doses of EPO have to be applied, which may induce undesired side effects such as polycythemia and secondary stroke [105, 106]. Research from our group reported that a TAT-derived peptide enhanced the capacity of EPO to cross the BBB in animals to at least two fold higher levels than unmodified EPO when intraperitoneally administered and up to five fold higher levels when intravenously administered. Not surprisingly, the therapeutic dose of the TAT fused EPO was about ten fold lower than regular EPO to achieve equivalent neuroprotection in terms of reducing infarct volume induced by MCAO in mice [108].

PROTEIN KINASES

δPKC is known to be activated by ischemia/reperfusion stress in multiple cell types of the brain [109] and is involved in cellular responses such as cell apoptosis, mitochondrial dysfunction, and oxidative stress [110, 111]. TAT-mediated delivery of δV1-1, a peptide inhibitor selective for δPKC, was shown to be protective even when given intraperitoneally 6 hours after onset of reperfusion following 2-hour MCAO [101]. Furthermore, TAT-δV1-1 improved microvascular pathology, characterized as an increased number of patent microvessels and increased cerebral blood flow following acute focal ischemia [101]. Indeed, TAT-δV1-1 fusion protein (also named KAI-9803) has already shown promise in a clinical trial of myocardial ischemia [112]. Clinical trials are now underway to test the effect of TAT-δV1-1 fusion protein on ischemic stroke.

In contrast to δPKC, the activation of εPKC, a different member of the PKC family, was documented to be neuroprotective against cerebral ischemia [113, 114]. Intravenous pretreatment with a fusion protein of TAT-ε-C- Kinase (RACK), a εPKC-selective peptide activator, 30 min prior to global cerebral ischemia conferred protection to the CA1 region of the rat hippocampus by regulating cerebral blood flow [114].

Protection against permanent or transient cerebral ischemia was also achieved when D-JNKI1 (also named XG102), a fusion protein containing a TAT sequence and a 20-amino acid sequence of the c-Jun N-terminal kinase (JNK) binding motif of Islet-Brain-1/JNK-interacting protein-1 (JIP-1), was applied intravenously or systemically with a 6-hour therapeutic window [115]. D-JNKI1- afforded neuroprotection is associated with caspase-3 activation and potent inhibition of phospho-c-Jun [116]. A subsequent study further showed that D-JNKI1 could also protect against ischemic damage in the presence of iPA [117].

POSTSYNAPTIC DENSITY-95 (PSD95) INHIBITOR

It has been known for a long time that activation of the NMDA receptor by pathologically high levels of excitatory neurotransmitter glutamate causes excitotoxicity and plays a key role in ischemic brain injury. However, direct targeting of the NMDA receptor is not practical for stroke treatment because blocking NMDA receptors causes substantial side effects in humans. Recent studies revealed that the postsynaptic density protein PSD95 is a prominent organizing protein coupling the NMDA receptor with other intracellular proteins and signaling enzymes including nNOS [118, 119]. A TAT coupled peptide (TAT-NR2B9c) targeting the NMDA receptor binding site on PSD95 has been shown to perturb the PSD95-NMDA receptor interaction. Intravenously or intraperitoneally injected TAT-NR2B9c crossed the BBB in both rats and mice [119] and potently alleviated ischemic brain damage in rats even when applied 3 hours after onset of permanent or transient MCAO. Behavioral studies showed that the reduction in infarct size was accompanied by improved long term neurological functions [120].

NEUROGLOBIN

Neuroglobin is an oxygen-binding protein found in cerebral neurons. Neuronal expression of neuroglobin enhances neuronal survival upon hypoxic challenge [121]. However, like most other protein neurotherapeutics, neuroglobin is too big to cross the BBB. Although intracerebral administration of a neuroglobin-expressing AAV vector has been shown to reduce infarct volume and improve functional outcome after focal ischemia, its invasive application and delayed expression after transduction render it clinically unfeasible. Recently, a TAT-fused neuroglobin has been developed and tested in the MCAO model of stroke [97]. TAT-neuroglobin fusion protein was delivered efficiently (at least 10-fold higher levels than endogenous neuroglobin) into mouse brain 4 hours after intravenous injection. Pretreatment with TAT-neuroglobin led to prominently reduced post-ischemic brain damage and improved neurological functions. Post-treatment with TAT-neuroglobin immediately after reperfusion, however, did not show significant protection against 2-hour ischemia.

OTHERS

Several other proteins such as Nogo-A extracellular peptide residues 1-40 (NEP1-40) [122, 123], heat shock protein 70 (HSP70) [124], and superoxide dismutase (SOD) [125] were show to penetrate the brain when fused with TAT and exert neuroprotective effects in models of cerebral ischemia. With ever-growing knowledge on the mechanism of neuronal damage in ischemic stroke and further improvements in the TAT transduction technique, greater numbers of therapeutic proteins or peptides will likely be tested against stroke treatment and eventually brought into the clinic for use in humans.

MECHANISMS OF CPP INTERNALIZATION

The mechanisms by which CPPs cross cell membranes are not completely understood. So far, three main entry mechanisms have been proposed: (1) direct penetration across the membrane; (2)
endocytosis-mediated entry; and (3) translocation through inverted micelle formation.

DIRECT PENETRATION

Early studies on the mechanism of CPP internalization suggested an energy-independent mechanism involving the direct translocation of CPPs across the cell membrane. Lowering the incubation temperature to 4°C or depletion of cellular ATP did not inhibit the rapid transduction of TAT into cells, suggesting that TAT-mediated protein transduction is endocytosis independent [22, 153, 154]. Other arguments in favor of an energy-independent mechanism include the absence of effect of metabolic or endocytosis inhibitors on CPPs internalization and the failure of radiolabeled CPPs to label endocytotic vesicles [155]. Moreover, structure-activity studies indicate that the internalization was equally effective whether the CPPs were in inverse or retro forms, which implies receptor-independent recognition [156]. A direct penetration mechanism was thus proposed which emphasizes the positive charges on most CPPs including TAT and Antp. It was suggested that the unfolded fusion protein interacts with the membrane through electrostatic interactions between the basic amino acids on CPPs and the negatively charged cell membrane polysaccharides or phospholipids, and then nucleates a pore, which enables protein translocation across the membrane [156-159]. According to this theory, once inside the cell, the fusion protein is refolded into its active form with the help of the endogenous chaperone system.

However, several studies have challenged the energy- and receptor-independent CPP translocation theory. It has been argued that artifactual uptake of CPPs arose following fixation procedures used for fluorescence microscopy. In addition, CPPs such as TAT or penetratin strongly bind to the plasma membrane and cannot be removed by the standard washing protocol used in FACS analysis. These membrane-bound peptides might be mistaken by FACS as internalized peptide, leading to questionable conclusions with respect to direct CPPs penetration [72]. The application of procedures avoiding these experimental artifacts yielded an alternative internalization mechanism favoring endocytosis-mediated entry (discussed below). However, there are still experiments supporting the direct penetration of TAT peptide across the membrane even in conditions devoid of the artifacts described above. For example, Ter-Avetisyan and colleagues demonstrated that a low temperature did not abrogate TAT uptake or change its intracellular distribution in live unfixed cells. They also showed that genetically engineered cells incapable of performing caveolin or clathrin-mediated endocytosis showed an equal capacity to take up TAT as control cells [160].

ENDOCYTOSIS-MEDIATED ENTRY

Endocytosis is a process of cellular ingestion by which patches of plasma membrane invaginate to engulf substances into the cell. Richard and colleagues demonstrated in their fluorescence microscopy study that live unfixed cells exhibit characteristic endosomal distribution of TAT or (Arg)₉. Flow cytometry analysis further indicated that the kinetics of TAT uptake were similar to the kinetics of endocytosis. These data are all consistent with the involvement of endocytosis in the cellular internalization of CPPs [72]. Endocytosis is now believed to be the major route for CPP internalization. There are three types of endocytosis: clathrin-mediated, caveoleae-mediated and macropinocytosis Fig. (2). Studies on TAT internalization with chemical inhibition of specific endocytotic pathways point to the involvement of multiple mechanisms. In support of lipid raft-mediated endocytosis involving caveoleae, Fittipaldi and colleagues showed that the internalization of TAT-EGFP endosomes was resistant to nonionic detergents and colocalized with caveoleae markers. Moreover, both the disruption of lipid rafts and the disturbance of caveolar trafficking impaired the internalization of TAT-GFP [161]. They also showed that conjugated TAT was not internalized in clathrin-coated endosomes. In contrast, using unconjugated TAT, Richard and colleagues showed that specific inhibitors of clathrin-dependent endocytosis partially inhibit TAT peptide uptake, while inhibitors of raft/caveolin-dependent pathway or genetic deficient of caveolin-1 expression

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**Fig. (2). Endocytosis-mediated CPP internalization.** Three types of endocytosis: clathrin-mediated, caveoleae-mediated and macropinocytosis, could mediate the internalization of CPPs.
could not inhibit the internalization of TAT [162]. Taken together, these results suggest that unconjugated TAT peptide might follow a clathrin-mediated endocytic pathway, whereas conjugated TAT follows a caveolae-mediated endocytic pathway. Furthermore, lipid raft-dependent macropinosytosis, yet another form of endocytosis that is independent of either caveolae or clathrin, was also implicated in the endocytosis of large TAT-fusion proteins in excess of 30kD [163] as well as unconjugated TAT [164]. Dose-dependent inhibition of TAT uptake was observed when cells were pretreated with amiloride, an inhibitor of the Na+/K+ exchanger required for macropinosytosis and when cholesterol was removed from the cell membrane [163, 164]. Indeed, the co-existence of different routes of endocytosis is not unique for TAT but true for many other CPPs such as homoarginine peptide [150, 165] and penetratin [134, 166].

Although endocytosis is an important mechanism for CPP-mediated cargo internalization, it is not surprising that this process of heteroepic internalization usually ends up with lysosomal delivery and significant degradation of CPPs and their cargoes. As a result, only a small fraction of the cargo reaches the cytoplasm in active form. In order to avoid such endosomal trapping, invasive approaches such as electroporation and microinjection were reported decades ago [167, 168]. Noninvasive methods were developed later to achieve efficient endosomal escape without physical damage to the targeted cells. One of these methods is to add peptides that can be activated in the acidic environment in the endosome and lyse the endosomal membrane to release entrapped CPP-cargoes. For example, HA2-TAT chimeric peptide, which consists of TAT and the pH-sensitive HA2 fusion peptide from influenza virus, has recently been engineered to enhance both macropinosytosis and endosomal release/delivery of TAT fused proteins and other macromolecules [163, 169].

**INVERTED MICELLE FORMATION**

An inverted micelle refers to an aggregate of colloidal surfactants where the hydrophilic groups are concentrated inside and the hydrophobic groups extend outward. The inverted micelle model was first proposed for the internalization of penetratin [156]. The model proposes that the binding of CPPs to negatively charged lipids in the cell membrane leads to the disruption of the lipid bilayer, resulting in the formation of inverted micelles that entrap the peptide in a hydrophilic environment. Further interaction of the peptides with membrane components leads to the inverse process, resulting in the release of peptides into the cell [156, 170]. Subsequent studies showed that inverted micelles are also involved in the internalization of arginine-rich CPPs [171].

Although endocytosis is now recognized as the predominant route of CPPs internalization, mounting evidence suggested that several different mechanisms may work in concert. It is possible that different CPPs utilize different internalization mechanisms depending on their specific biophysical properties, the nature of their cargo and the experimental conditions. Furthermore, one type of CPP may use several different pathways simultaneously for efficient internalization.

**ADVANCES IN CPPS’ DELIVERY SYSTEM**

**Organelle-targeted Delivery**

Organelle-specific CPPs are under rapid development to provide a means to target therapeutic agents not only to the cell, but also to a specific subcellular compartment to achieve optimal activity and reduce non-specific effects. So far, the nucleus and the mitochondria have been successfully targeted with CPPs for gene therapies and anti-cancer chemotherapies.

Nucleus-targeted delivery is important for gene therapies. A common strategy to improve nuclear delivery of CPP-fused cargoes is to add a nuclear localization signal (NLS) to the conjugates. NLS-conjugates are highly cationic short peptides. The sequence PKKKRKV derived from SV40 large T antigens is the most often used NLS. For example, this NLS sequence is a component of MPG chimeric CPP and has been shown to improve the nuclear uptake of plasmid DNA and oligonucleotides without necessitating nuclear membrane breakdown [172]. The NLS-mediated nuclear delivery system has been mainly tested in tumor cell lines or in vivo tumors because a large number of anticancer drugs are DNA toxins that must bind nuclear DNA or its associated enzymes for cytotoxic effects. Recently, a nucleolin binding peptide (NBP) was reported to deliver macromolecules including fluorophores, recombinant protein and DNA to the nuclei of ocular tissues in vivo. Pegylated NBP nanoparticles could further improve nuclear delivery and the expression of transgenes. This NBP might be a useful tool for therapeutic delivery to the eyes [173]. Future studies are warranted to evaluate the potential of nuclear-targeted CPPs in gene therapies against brain diseases, such as gene therapy for enzyme/neurotransmitter replacement in neurodegenerative diseases, antisense gene therapy for brain cancers, and anti-apoptotic/anti-oxidative gene therapy for other brain injuries such as stroke.

Mitochondria are another subcellular compartment specifically targeted by modified CPPs. Mitochondria are a primary source of reactive oxygen species (ROS) in the cell, and release critical apoptotic signaling molecules when damaged. Mitochondria-specific CPPs may thus provide a platform for anti-apoptotic or antioxidant therapies for many common diseases. One strategy to target mitochondria is to incorporate a mitochondrial signal sequence, such as mitochondrial malate dehydrogenase (mMDH) signal sequence into a CPP fusion protein [174]. For instance, it has been shown that TAT-mMDH-GFP was enriched in mitochondria in vitro and detectable throughout fetal and neonatal pups when injected into pregnant mice [174]. Recently, mitochondria penetrating peptides (MPP) were designed by Horton and colleagues. They examined a panel of mitochondrial transporters-synthetic cell-permeable peptides and observed efficient mitochondrial penetration in a variety of cell types [175]. According to their studies, MPP efficiency can be optimized by altering lipophilicity and charge. The application of mitochondria-specific delivery to neuronal cells was reported by Zhao and colleagues, who described that a novel mitochondria-targeted CPP antioxidant SS-31 (D-Arg-Dmt-Lys-Phe-NH2; Dmt = 2',6'-dimethyltyrosine) protected against oxidant-induced mitochondrial dysfunction and apoptosis in two neuronal cell lines [176].

**The Combined Use of Peptides and other Transportation Mechanisms**

Many new formulations of CPPs combined with other transportation mechanisms have increased CNS delivery capacity. For example, liposomes formulated with TAT-conjugated cholesterol were recently shown to exhibit high delivery efficiency across the BBB [144]. In addition, NPs conjugated with low-molecular-weight protamine (LMWP), a CPP with high cell-translocation potency, exhibited enhanced cellular accumulation compared to unmodified NPs and increased the efficiency of brain delivery of coumarin after intranasal administration [177]. In another study, TAT was used to facilitate transport of fluorescein-labeled silica NPs into the brain [143]. These studies suggested that CPP-conjugated NPs are effective carriers for brain drug delivery. With the increased interest in peptide-based drugs for the treatment and diagnosis of CNS diseases, CPP-conjugated peptidic NPs may become increasingly utilized as nanoplatforms to improve transportation across the BBB.

**LIMITATIONS**

**Lack of Tissue and Cell Selectivity**

The lack of both tissue and cellular phenotype specificity for cargo delivery represent important caveats for using CPPs technology in the nervous system, where inappropriate targeting could lead to dire consequences for neurological function and animal behavior. Although there is a tendency for TAT-fusion proteins to favorably
transduce neurons over glial cells [43], this delivery system is still unable to convey proteins specifically to a targeted cell type within the CNS or even to the CNS as a whole. In this regard, a double-targeted delivery system simultaneously capable of extracellular accumulation at the appropriate cellular location through specific receptors or enzymes, and intracellular penetration through CPPs may overcome this shortcoming and render CPPs capable of distinguishing between non-target and target cells. For example, lipoplexes modified with monoclonal anti-myosin monoclonal antibody specific toward cardiac myosin and with TAT for cell penetrating can achieve targeted gene delivery to ischemic myocardium [178]. Targeted CPP delivery system to CNS cells, however, has not yet been developed, but will be essential before clinical translation is feasible.

Uncertainty in Fusion Protein Activity

Another caveat of using CPP as the delivery system is that there is no guarantee that the transduced fusion protein will gain its natural biological activity in target cells. Once inside the cells, it has been proposed that the CPP-fusion protein is properly refolded by chaperone proteins such as HSP 90 [179]. However, we found that the TAT system may not deliver all classes of proteins with equal success. For example, proteins that are only biologically active in their multimeric state may not be good candidates for this approach. Even the delivery of native monomeric subunits cannot ensure the proper assembly of the subunits into a functional enzyme. In this regard, the CPPs delivery system has not yet rendered the viral vectors for delivery of gene products obsolete. This is because, in many instances, the viral system utilizes host machinery for endogenous folding, enzyme assembly or post-translational modifications in a more efficient and appropriate manner.

Stability

For successful in vivo delivery, the CPPs must also not be metabolically degraded until they send their cargo to the appropriate destination. However, concerns about the stability of these peptide vectors still plague the field. For instance, the free TAT CPP has six potential trypsin cleavage sites [180] and thus has a short half-life of just a few minutes in the presence of trypsin [181]. Several structure-activity relationship features, including amino acid sequences, amino acid three-dimensional configurations, incorporation of non-α-amino acids, and type of linkage between CPPs and cargoes, have been shown to affect the stability of CPP conjugates in human serum and within cells. Generally, the D-configuration of the peptide is less sensitive to degradation by proteases and remains intact in serum and cells for a much longer time than the naturally occurring L-amino acids [182]. The replacement of protease-sensitive amino acids with their stable synthetic mimics [183, 184] or the insertion of β-alanines [182] also increase CPP stability. A recent study showed that the shielding of TAT through TAT conjugated poly(ethylene glycol)-phosphatidyl ethanolamine block copolymer (TAT-PEG-PE) incorporation into PEG-PE micelles could protect TAT against trypsinolysis with an over 100-fold increase in half-life compared to free TAT [181]. With all our efforts to stabilize CPPs, one must also keep in mind that the metabolic cleavage of the CPPs after internalization is a prerequisite for the release of their cargo. Another tradeoff of gaining enhanced metabolic stability is an impairment in the clearance of CPPs and a subsequent increase in their toxicity. Therefore, a thorough evaluation of each modified CPP, with an emphasis on safety profiles, as discussed below, is essential for the eventual translation of these CPPs into the clinic.

Toxicity and Immunogenicity

Various CPPs have toxic effects on cultured cells. A comprehensive characterization of the metabolic effects of CPPs was done by Kilk et al [185]. Their study suggested that transportan treatment changed the GSH redox ratio and significantly decreased cellular redox potential, while other four tested peptides (TAT, Antp, Arginine9, and MAP) showed minimal effect on metabolic profiles. Cardozo and colleagues showed that although up to 100 μM TAT (48–57) alone was essentially harmless, TAT-peptide conjugates triggered significant cytotoxicity when used at concentrations higher than 10 μM [186]. In comparison, free penetratin exhibits intrinsic toxicity and penetratin conjugates showed more cellular toxicity than TAT conjugates. Although all the factors that determine the cytotoxicity of CPPs are still not completely clear, peptide length, peptide concentration, cargo molecule and coupling strategy have all been suggested as contributing factors [187].

The immunogenicity of CPPs is not frequently reported. However, since most CPPs are derived from non-human proteins, they have the potential to induce immune responses when used in a clinical scenario [188]. Therefore, further examinations of immune responses to CPPs are highly warranted.

CONCLUSIONS

On the one hand, the BBB is essential to maintain the integrity of the brain and the spinal cord, and yet it also presents a vexing barrier to getting drugs to their neural destinations in human CNS diseases. What was first observed by Paul Ehrlich as an inability of vital dyes to cross into the brain has therefore motivated many researchers in the past century to overcome this formidable obstacle to CNS drug delivery. Relative to previous years, the past decade witnessed significant achievements in successfully penetrating the BBB and facilitating targeted CNS drug delivery to injured neurons, to tumors, and even to specific subcellular organelles. This is largely due to the noninvasive drug carriers, especially CPPs, which show great potential to deliver various neurotherapeutics across the BBB for the treatment of ischemic brain injury, among other conditions. However, it behooves us to retain a balanced view of these new techniques because certain weaknesses, in particular the lack of tissue and cell type specificity, still restrict their clinical application. Future modifications of CPP sequences or the combinations of CPPs with other drug carriers are expected to improve the specificity of CPPs as well as reduce the side effects associated with the delivery of cargoes to unintended organs. With such modifications in hand, CPP research may lead the way to novel avenues of non-invasive stroke management in humans.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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