# Agile Delivery of Protein Therapeutics to CNS

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## Abstract

A variety of therapeutic proteins have shown potential to treat central nervous system (CNS) disorders. Challenge to deliver these protein molecules to the brain is well known. Proteins administered through parenteral routes are often excluded from the brain because of their poor bioavailability and the existence of the blood-brain barrier (BBB). Barriers also exist to proteins administered through non-parenteral routes that bypass the BBB. Several strategies have shown promise in delivering proteins to the brain. This review, ***first***, describes the physiology and pathology of the BBB that underscore the rationale and needs of each strategy to be applied. ***Second***, major classes of protein therapeutics along with some key factors that affect their delivery outcomes are presented. ***Third***, different routes of protein administration (parenteral, central intracerebroventricular and intraparenchymal, intranasal and intrathecal) are discussed along with key barriers to CNS delivery associated with each route. ***Finally***, current delivery strategies involving chemical modification of proteins and use of particle-based carriers are overviewed using examples from literature and our own work. Whereas most of these studies are in the early stage, some provide proof of mechanism of increased protein delivery to the brain in relevant models of CNS diseases, while in few cases proof of concept had been attained in clinical studies. This review will be useful to broad audience of students, academicians and industry professionals who consider critical issues of protein delivery to the brain and aim developing and studying effective brain delivery systems for protein therapeutics.

## Keywords

protein therapeutics; central nervous system; blood-brain barrier; protein delivery; administration route; brain delivery

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## 1. Introduction

Protein therapeutics has made significant progress during the past 30 years, beginning with the invention of the first recombinant protein used in clinical practice, a human insulin [1]. Since then, development of protein therapeutics has been one of the biotech's most notable successes. In recent years, the number of protein-based therapeutics reaching the marketplace has increased exponentially. As of today, more than 130 proteins or peptides are used in clinics and many more are in development [2]. The currently marketed proteins include enzymes, antibodies, clotting factors, anticoagulants, modern insulins, growth hormone, follicle-stimulating hormone, hematopoietic growth factors, interferons, interleukins and others. The market of the therapeutic proteins holds tremendous potential for future growth and it is estimated that by the end of 2018, it may reach the mark of US $165 billion as new products may enter the sector. As patents on first-generation proteins wind down, the industry seeks to protect their markets by introducing protein delivery technologies that provide for improved stability, bioavailability and safety of the therapeutic proteins. Such technologies aim to overcome obstacles to the clinical application of the proteins due to a lack of desirable attributes for adequate absorption or distribution. It therefore becomes critical to incorporate proteins in safe, stable and efficacious delivery systems. Because proteins face formidable enzymatic and penetration barriers, efficient protein delivery to its destination in the body remains a very challenging if not a formidable task.

There is a tremendous potential to develop protein therapeutics for the treatment of neurological and neurodegenerative disorders. Examples include Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), human immunodeficiency virus 1 (HIV-1)-associated dementia (HAD) (or more generally HIV-associated cognitive dysfunction), multiple sclerosis (MS), lysosomal storage disorders (LSDs; Gaucher's disease, Niemann-Pick disease, Tay-Sachs disease and Sandhoff's disease, Krabbe's disease, Fabry's disease, metachromatic leukodystrophy amongst nearly 50 total disorders) and others. Other diseases associated with the central nervous system (CNS) include brain tumors, stroke, traumatic brain injury (TBI), and metabolic disorders. Some examples of potential protein therapeutics to treat these CNS related disorders include enzymes in LSDs, antibodies in AD and brain tumors, neurotrophic factors in PD and stroke, and gut-brain hormones in obesity.

Clinical use of these proteins, however, is extremely challenging because of the unique and complex environment imposed by the CNS. Systemic delivery of proteins to the brain inevitably encounters two major hurdles: the rapid serum clearance and the limited penetration at the blood-brain barrier (BBB). Some protein molecules, such as neurotrophic factors can cross the BBB to some extent but are rapidly cleared from the blood, whereas others, such as antibodies, are stable and long circulating in blood but absolutely not permeable at the BBB. In both cases systemic delivery of proteins does not allow to attain their sufficient brain concentration for effective treatment. Proteins can also access the brain through alternative delivery routes that allow bypassing the BBB, such as intracerebroventricular (i.c.v.), intraparenchymal, intranasal (i.n.) or intrathecal (i.t.) administration. However, in most cases the brain uptake of proteins following such

administration routes is still surprisingly low, especially in the targeted brain regions where protein therapeutics needs to be delivered. It has been gradually accepted that serious biological barriers are associated with each of these alternative delivery routes.

Therefore a great deal of effort has been dedicated to developing the drug delivery systems and approaches that could help protein molecules crossing numerous barriers on their way to the site of action in the brain. Multiple drug delivery strategies were explored in the attempts to address this challenge. For example, chemical modification of proteins with poly(ethylene glycol) (PEG), known as PEGylation [3], or incorporation of proteins into poly(D,L-lactic-co-glycolide) (PLGA) particles [4, 5] increased stability and bioavailability of certain proteins and resulted in development of the Food and Drug Administration (FDA) approved products for various peripheral diseases. However, neither of these technologies has shown much promise so far in delivering protein therapeutics to the brain for treatment of CNS related diseases. Several specific molecules (antibodies, peptides, etc.) that can target and cross BBB through intrinsic transport systems available in brain endothelium were identified and conjugated to protein of interest to create targeted therapeutic agents for CNS related diseases. However, no such conjugate has progressed far enough to enter clinical trials although similar conjugates with small molecule drugs seem to be somewhat more advanced (e.g. paclitaxel-Bp-2 ANG1005, Angichem, Inc). Some of the studies in this area go back nearly 30 years, and yet during this considerable period, despite consistent and steady effort by numerous capable researchers across the globe relatively little progress was achieved, which only underscores the enormity of the task.

However, analysis of previous experience in this field along with understanding of the recent achievements and trends in the drug delivery and nanomedicine science allow us to suggest that a new explosive development is just behind the corner. We believe that investigators should expect a very exciting journey during the next decade in pursuit of novel CNS technologies and therapeutics and that a critical mass of knowledge has been reached enabling new principal breakthroughs. In anticipation of this development we decided to critically analyze the past experiences from the current prospective that in our view in essential to achieve success in this field. We believe that the recent dramatic improvement in understanding the molecular physiology of CNS environment and the various barriers that exist on the way of successful protein delivery to the brain will be conductive to future progress. There is growing realization that the BBB, as part of the neurovascular unit (NVU), represents an interactive, dynamic, regulatory interface between the CNS and peripheral tissues [6–15]. It is also clear that various pathological processes associated with neurological and neurodegenerative disorders alter the NVU and cause BBB dysfunction, which brings some opportunities and challenges to the design of protein therapeutics for these disorders. The choice of the routes of administration of these therapeutics is also pivotal and requires consideration of the disease stage (chronic or acute), location within the brain (widespread or local), and chemical nature of the compound to be delivered. We also believe, that there is a great opportunity in using nanomedicine approaches to improve the site-specific delivery and brain regional distribution of proteins administered though non-conventional routes allowing to avoid the BBB. It should be noted that due to small amounts of substances that can enter the brain, robust and reliable bioanalytical assays are needed for the analysis of the pharmacokinetics (PK) and

biodistribution of the protein therapeutics. Carefully designed PK studies and proper interpretation involving analysis of PK and pharmacodynamics correlations and dose-responses are absolutely essential. Development of animal models that closely recapitulate human diseases and understanding of the limitations of these models are needed to carefully interpret results of the preclinical animal studies and use these results as for guidance for clinical trials. Here we present the readers with this review which briefly and sequentially considers the 1) BBB physiology and pathology in CNS related disorders; 2) main classes of protein and peptide therapeutics for CNS; 3) delivery routes for protein therapeutics; 4) chemical modification of proteins for CNS delivery; and 5) particle-based carriers for CNS delivery of proteins. We hope to disseminate and advance an in-depth understanding of each of these strategies and provide useful information for future design of protein delivery to the brain.

## 2. BBB physiology and pathology in CNS related disorders

Discovery of BBB is usually ascribed to the work of Paul Ehrlich and Edwin Goldman over a hundred years ago. They observed that intravenously injected dye stained all the organs with the exception of the brain and that the same dye exclusively stained the brain after injection into the brain [16, 17]. Thomas Reese and Morris Karnowsky further demonstrated that the blood was separated from the brain at the level of brain microvessel endothelial cells (BMECs). Under high resolution electron microscopy it was shown that intravenously injected horseradish peroxidase (HRP), 43 kDa, stained only BMECs and the tight junctions (TJ) between BMECs but was not detectable beyond the vascular endothelium [18, 19]. Accordingly, the physiological BBB generally refers to the continuous layer of BMECs [20] (Figure 1). Different from the capillaries of peripheral tissues, BMECs are sealed by TJ, virtually excluding paracellular transport of any molecule from blood to brain. It is also characterized by 1) small number of vesicles at the luminal side of BMECs, 2) presence of the drug efflux pumps at the basal luminal side, and 3) high metabolic activity responsible for degradation of most internalized substances. Altogether these morphological and functional features result in limited transcytosis and endocytosis and thus explain why BBB acts as a formidable barrier for many substances to enter the brain. Adjacent to the brain capillaries along the basal luminal are perivascular cells (also called pericytes) which are now recognized to play important roles in the regulation of CNS homeostasis, the BBB integrity, the macrophage activity and modulation of blood flow [21]. A thin basement membrane (i.e. basal lamina) supports the abluminal surface of the endothelium surrounding the endothelial cells and pericytes. Another important cell type involved in the BBB function in the brain is astrocytes. One type of brain astrocytes completely envelops BMECs and pericytes with their end feet and this structure initially was thought to play a substantial role by aiding the maintenance of the BBB. However, more recent studies show that astrocytes are more important in the regulation of water and ion homeostasis and secretion of chemokines as a sensor of pathologic changes, which helps modulating the BBB function [22]. Along with adjacent glia and neurons, BMECs, pericytes, and astrocytes comprise NVU, a dynamic cellular interface that defines the functioning of the CNS [23].

Pathology of many neurological disorders, including AD and HIV-associated cognitive dysfunction, as well as stroke, brain tumors, and TBI involves BBB dysfunction (Figure 1)

[6–15]. Failure of the BBB due to alteration of the TJ in many cases, like stroke or TBI, is a direct result of the disease pathology, while in other cases, like MS, it is a precipitous event of the disease [23]. Sometimes, like in the case of AD the BBB contributes to disease onset and progression and thus itself becomes a therapeutic target [24]. There are reports that the BBB could be compromised and associated with the neuropathology and disease deterioration in LSDs [13]. Disruption of the TJ of the BBB as a result of the disease-induced hypoxia has been thoroughly investigated [25, 26]. Disease-induced inflammation also contributes to the alteration of the BBB. Abnormal, monocyte migration across the BBB to the brain is probably induced by a release of inflammatory cytokines during pathological processes associated with the disease progression [27]. Structural and morphological alterations in the NVU and BBB associated with the disease, such as basal lamina degradation by metalloproteases in hemorrhagic transformation after human ischemic stroke were also described [28, 29]. Likewise, during ALS the BBB impairment was accompanied by a decreased mRNA expression of ZO-1 and occludin as well as agrin, a basal membrane constituent [30]. Disease may also affect functional components of BBB responsible for transport of various substances in and out of the brain. For example, increased number of pinocytic vesicles in the BMECs has been described in certain cases such as AD [31] HIV-related neuropathologies [32], some leukodystrophies [33], and exposure of the brain to microwave radiation [34]. Both down and upregulation of the expression of the P-glycoprotein (Pgp), a major outwardly drug efflux transporter at the luminal membrane of BMECs was observed during brain inflammation, oxidative stress, and HIV-1 infection [35]. In some cases the malfunction of the BBB transport systems is directly linked to the disease cause. For example, in obese patients function of BBB transporter of leptin, a peptide hormone that controls appetite and metabolism, is impaired. Leptin, is produced by fat tissues and reaches its target in the brain by crossing BBB via specific, saturable transport system in healthy individuals [36]. Inability to cross BBB is referred to as leptin resistance and results in metabolic dysfunction, which needs to be overcome to deliver sufficient amount of leptin to the brain [37–40].

Clearly, it is important to recognize and understand the pathological changes of the BBB when designing the delivery system for protein therapeutics to access the brain. However, one cannot simply rely on the breakdown of the BBB. First, not all neurological disorders are characterized by BBB dysfunction. In rat model of parkinsonism no changes in BBB functionality was observed and it was concluded that BBB dysfunction was not a specific characteristic of this disease [41]. Second, even in the cases when the BBB opening as a result of the disease was documented the extent of the opening remains unclear, as different results were obtained using low molecular mass and polymeric probes [42–45]. Third, the timing of the opening and its relation to the disease progression in many cases is not well understood [46–49]. Both the extent of the BBB opening and the timing, may be dependent on the disease progression and the animal model used to study these phenomena. For example, it is well known that the brain tumor vasculature is characterized by altered BBB. However, the nature and extent of these changes is defined by type of tumor. In low-grade astrocytomas barrier properties of tumor endothelium are close to normal, while in high-grade astrocytomas, glioblastomas, and brain metastases of systemic cancers the vasculature is leaky and lacks differentiated transport properties of normal brain endothelium [50].

Finally, it is unclear whether the areas of the brain that become accessible due to the BBB breakdown are really in need of delivery of a therapeutic agent, or if the therapies should be delivered to the brain areas, which not affected by the disease pathology to the extent resulting in the BBB compromise.

## 3. Main classes of protein and peptide therapeutics for CNS

Current efforts in development of CNS biotherapeutics have focused on several classes of molecules including gut-brain hormones, lysosomal enzymes, neurotrophic factors, antibodies, and peptides. Some of the proteins and peptides evaluated for various neurological disorder indications in patients or approved for clinical use are listed in Table 1. All these molecules are believed to act upon targets in the CNS, which underscores the importance of their delivery to the brain. One class is gut-brain hormones that are released from the gut and exert their biological function partially or exclusively within the CNS. These hormones along with circulating nutrients and neuronal peptides modulate appetite, feeding and metabolism. Accordingly, they provide a range of therapeutic opportunities for treatment of metabolic disorders (e.g. diabetes, obesity) [76]. All the gut-brain hormones are acidic, less than 5 kDa in size (except for leptin, which is 16 kDa), they easily degrade and rapidly clear from circulation through renal clearance. As a result they have poor bioavailability after systemic administration, which otherwise would be most convenient and acceptable for these treatment needs (chronic, non-life threatening diseases, multiple dosing, patients self-use). Although hormones with peripheral site of action can certainly benefit from increasing their circulation time, most gut-brain hormones need to cross the BBB to exert physiological effect. In some cases, for example, leptin, the disease condition is associated with the impairment of a normal physiological transport mechanism at the BBB and therefore increasing permeability of such molecules at the BBB becomes crucial to ensure physiological effect [77, 78].

Lysosomal enzymes represent another major category of proteins that need to be delivered to the brain for treatment of LSDs that manifest neurological pathologies. There has been considerable success in development of enzyme replacement therapies (ERT) of LSDs with peripheral manifestations, such as type 1 Gaucher disease, Fabry disease, and Pompe disease [79]. For such diseases FDA has approved at least nine systemically administered ERT drugs. However, systemic therapies for LSDs associated with CNS pathologies appear to be less effective possibly due to limited penetration of the potential therapeutic agents at the BBB. Many lysosomal enzymes are homodimeric or heterodimeric glycoproteins of relatively large molecular weight (>100 kDa). Almost all of them are tagged with mannose 6-phosphate (M6P) moieties, which ensure their lysosome sorting in the cells [80]. Unfortunately, the M6P-receptor is not expressed in brain endothelium in physiological conditions and cannot be used to target proteins to the BBB [81–83]. To the contrary, circulating enzymes are readily engulfed by macrophages via M6R receptor and thus are rapidly cleared from the blood [84]. Fast clearance, large molecular weight, hydrophilic nature arising from the attached glycans, and resulting inability to penetrate BBB are all obstacles to effective delivery of lysosomal enzymes to the CNS through systemic routes. Indeed one promising example of ERT for CNS related LSDs, evaluated in clinical trials involves treatment of mucopolysaccharidosis II (MPS II) patients with iduronate-2-sulfatase

(I2S) through i.t. administration [60, 61]. Therefore, bypassing the BBB by choosing an appropriate administration route as discussed below could be a valuable approach to delivery of such therapeutic agents.

Neurotrophic factors also known as "neurotrophins" represent family of proteins that are responsible for the growth and survival of developing neurons and the maintenance of mature neurons functionality. Examples include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), neurotrophin-3 (NT-3), fibroblast growth factor (FGF) and epidermal growth factor (EGF). These proteins commonly carry a positive charge (pI > 8), have a molecular weight ranging from 5 to 30 kDa and exhibit potency at femtomolar to nanomolar concentrations [85]. Albeit neurotrophins have long been explored as potential neuroregenerative and neuroprotective therapeutic agents during various CNS-pathologies and some were tested in clinical trials, none of them have emerged as regulatory approved medicines. Interestingly, an active transport mechanism for some of neurotropic factors such as NGF, NGF and NT-3 does exist at the BBB allowing for transport of systemic proteins to the brain [86, 87]. However, these agents still face multiple challenges to CNS delivery that are common for most CNS therapeutics, including low rapid enzymatic inactivation, multiple clearance processes, sequestration by serum proteins and peripheral tissues, and immunogenicity. Furthermore, there is an additional challenge due to a need of targeting a neurotrophic drug to a brain region relevant to its pathology. Indeed neurotrophin receptors and signaling pathways are spread throughout the brain where they play multiple physiological roles in impacting cell death, plasticity and survival. Thus there is a possibility for off-target effects unless neurotrophins are delivered precisely to their needed sites of action. On the other hand missing the proper point of delivery could result in the lack of the needed activity. Therefore, developing methods allowing for delivery of neurotrophins to specific brain regions is especially important. Examples, include GDNF treatment of PD patients, where a series of clinical trials have shown contradictory or negative effects possibly due to insufficient penetration of this neurotrophin to its site of action [64, 65, 88] and new delivery methods are designed specifically to address this challenge.

Brain delivery of antibodies is currently mainly investigated for treating AD and brain tumors [89–93]. However, possibilities for antibody-based CNS therapeutics are enormous. A relatively long serum half-life, high potency, and limited off-target toxicity are all very desirable pharmacological advantages of antibodies for developing CNS therapeutics. However, due to a large molecular mass (e.g. 150 kDa for IgG), antibodies are generally restricted to extracellular space. Accordingly, whereas bioavailability is a much lesser concern for antibody delivery compared to other proteins, low permeability of antibodies at the BBB is a major issue that has been researched since early 1990s [94]. Some of the approaches to antibody delivery are considered below in Section 5.4, but generally these approaches has not yet not evolved to reach the clinical stage and efficient delivery of the antibodies to the brain remains a major unmet medical need.

"Peptides" are short chains of amino acid monomers linked by amide bonds and are considered here as a separate category due to their relatively small size (less than 20 amino acids and molecular mass less than 5 kDa) and common lack of secondary structure. They

can function as receptor agonists or antagonists by activating or blocking central signaling pathways, and depending on the structure, can be used in treatment of numerous CNS related disorders, such as, schizophrenia, anxiety, depression, autism and others. Therapeutic peptides offer multiple advantages compared to small molecules: high specificity and potency, minimal cross-reactivity, no tissue accumulation, efficient metabolism, limited drug–drug interaction, minimal side effects and low immunogenicity. However, similar to most proteins, peptides exhibit very short serum half-life (min. to hrs.), low serum stability and poor permeability at the BBB, which hinder therapeutic use of these molecules for treatment of CNS disorders. Attempts to address these issues have involved various manipulations with the conformation and molecular structure of peptides as reviewed elsewhere [95]. Moreover, unlike most proteins discussed herein some especially lipophilic and short sequence peptides, are substrates of drug efflux transport system, Pgp overexpressed at the BBB [96]. Therefore bypassing or inhibiting this transport system is important for the successful delivery of such molecules.

Outside of the scope of this review are synthetic peptides and proteins used in vaccination, some of which have shown considerable promise in treating CNS related diseases. For example, a therapeutic vaccine, CDX-110 consisting of 14 amino acid peptide, rindopepimut conjugated to immunostimulator molecule, keyhole limpet hemocyanin (KLH) can activate anti-tumor immune response by targeting tumor-specific epidermal growth factor receptor, EGF receptor (EGFR) vIII. Both preclinical and clinical studies demonstrated that following intradermal injection, CDX-110 is effective in treating glioblastoma multiforme, the deadliest form of brain cancer [97, 98]. Another therapeutic vaccine PD01A, designed to target the alpha-synuclein tangles, an aggregated protein associated with pathological cell death in PD, has entered phase I trials [99, 100]. Extensive studies have shown potential of passive and active immunization for the treatment of AD [92, 93]. However, peptide or protein vaccines usually target the peripheral immune system rather than the brain. Therefore, successes in these vaccines development depend on different set of factors than those defining successes of CNS therapeutics. Hence we will not refer to vaccines in the context of our further consideration.

## 4. Delivery routes for protein therapeutics

The path of a therapeutic agent to its target organ and tissue begins at the site where the molecule is given to the body. Most current protein therapeutics including FDA approved products (e.g., antibodies and hormones) are administered by parenteral injection into fat tissue (subcutaneous, (s.c.)), muscles (intramuscular, (i.m.)) or veins (intravenous, (i.v.)). In few cases enteral and pulmonary routes were also explored to deliver protein therapeutics that require frequent dosing to attain the therapeutic window [101–103]. For example, oral delivery, one of the most convenient and least expensive enteral administration routes is used for non-CNS targeted therapeutic polypeptides such as insulin, calcitonin, interferons and human growth hormone [104]. Thee oral bioavailability of polypeptides can be further increased by their encapsulation into liposomes or nanoparticles or/and using permeation enhancers [105–108]. However, the oral route is nearly impossible for CNS therapeutics, because of the presence of two formidable barriers - the gastrointestinal lumen and BBB.

Early approaches to delivery of protein therapeutics to CNS mainly focused on traditional parenteral injection routes - i.v., i.m. and s.c. In addition, there is a long history of using the central administration routes, i.c.v. and intraparenchymal, that bypass the BBB to deliver proteins to CNS, in particular, during life-threatening conditions (Figure 2). More recently, a non-invasive administration at the nasal cavity has attracted considerable attention and shown promise in delivery of therapeutics to the brain. Another way to bypass the BBB during delivery of therapeutics is to use i.t. route, an approach that has attracted attention in treatment of CNS disorders because of its relatively low invasiveness compared to the central administration. A comprehensive description and consideration of the administration routes is beyond the scope of the current review. However, it is useful to describe some general principles of these routes to determine potential opportunities and challenges for delivery of protein therapeutics to the CNS.

## 4.1 Parenteral administration

The substance delivered by traditional parenteral injection (i.v., i.m., or s.c.) can directly access systemic veins by bypassing the portal veins and hepatic first-pass elimination and ultimately be distributed to the whole body including the brain through the vascular system. A unique advantage of the systemic vascular route is the presence of the dense network of brain capillaries. These capillaries theoretically can allow for an instantaneous and even distribution of a therapeutic agent to the closest surrounding cells of the brain, which could be of great benefit for the treatment of CNS disorders that display widespread pathology or have unknown location in the brain. Evidently, an effective treatment using parenteral route relies on the ability to deliver effective amount of a therapeutic agent from the blood to the brain disease sites. This is often challenged by poor serum bioavailability of a therapeutic agent (rapid serum clearance, binding to serum proteins, degradation, and tissue distribution) and the existence of the BBB. Finally, the bioavailability of a therapeutic agent in the brain interstitial fluid is affected by the clearance of this agent from the cerebrospinal fluid (CSF) and degradation of this agent in extracellular space (ECS). All these factors collectively contribute to the actual concentration of therapeutics within the brain and a delivery system needs to be designed to maximize the efficiency at each step.

Notably, certain macromolecules that can circulate long in the blood and remain stable in circulation were detected in the brain in spite of their poor penetration at the BBB. For example, albumin, a BBB impermeable molecule, was detected in CSF at CSF/serum ratio of 0.5% [109]. The antibodies against amyloid β (Aβ) protein and erythropoietin both having long half-life in the blood, were shown to reach the brain at sufficient amounts to be effective in brain disease models [110–114]. The entry of these molecules to the brain is believed to be associated with *an extracellular pathway* due to the functional leakiness of the BBB, which could occur at a number of sites within the CNS, including the circumventricular organs, the arachnoid space/pial surface and the nasal epithelium [115, 116]. This extracellular pathway at BBB can be described by a nonsaturable and dose-dependent uptake mechanism at least in the case of IgG [117]. Protein therapeutics candidates may enter the brain using similar mechanisms. The good candidates for this route are the molecules characterized by a small volume of distribution, high potency in the CNS, and absence of brain-to-blood efflux that could efficiently decrease their brain

concentration. For example, an i.v. infusion of a high dose of recombinant human β-glucuronidase over long duration resulted in a brain uptake of this protein accompanied by reduction in toxic substrate storage in central neuronal lysosomes in a mucopolysaccharidosis VII mouse model [118]. Various methods were developed to increase serum bioavailability of proteins, such as their conjugation with hydrophilic polymers like PEG (PEGylation), or encapsulation of in micro- and nano-size particles [2, 119]. From a delivery viewpoint, these methods can increase the blood circulation time and serum stability of the delivered proteins. Accordingly, they could be benefit CNS delivery of proteins provided that the delivered materials 1) can still exploit the extracellular pathways, and 2) remain active in the CNS (or in the case of the nanocarriers are released into the brain). The key issue, however, is that diffusion of serum macromolecules to the brain via extracellular pathways is severely limited. Even in most pathological conditions that may be associated with some leakiness and/or "opening" of the BBB these pathways are not sufficient to secure a robust pharmacodynamic response. Therefore, in most cases, increasing transcellular permeability at the BBB is critical to overall improvement of the parenteral delivery and efficacy of a biotherapeutic agent in the CNS.

Relatively little attention was devoted to improving the bioavailability of therapeutic agents in the brain. It is probably true that the molecules with increased serum bioavailability would also be better preserved in brain interstitium and ECS. However, it is not clear whether a delivery system that improves peripheral bioavailability of therapeutics also remains intact after crossing the BBB. Justin Hanes's laboratory has recently reported that densely coated PEG nanoparticles over 100 nm can diffuse in brain parenchyma ECS [120]. This suggests at least a theoretical possibility of designing a nanoscale size delivery system that after crossing the BBB can continue its journey through ECS to the target cell within the brain.

### 4.2 Inctracerebroventricular infusion

The administration of proteins through i.c.v infusion allows these proteins to bypass the BBB, directly enter the lateral ventricles and circulate within the ventricular and extraventricular CSF. However, the clinical trials of i.c.v protein therapeutics have been rather disappointing. For example, in one trial the NGF was given i.c.v. to 3 AD patients [62]. Three months after this treatment a significant increase in nicotine binding in several brain areas in the first 2 patients and in the hippocampus in the third patient were observed. However, a clear cognitive amelioration could not be demonstrated. Moreover, the treatment resulted in significant adverse effects such as back pain and body weight loss, which strongly diminished enthusiasm about the potential of this treatment [62, 121]. In another clinical trial the GDNF was administered i.c.v. to PD patients [88]. This treatment did not result in any positive response, although no significant side effects were observed either. Subsequent trials of GDNF in PD patients also produced contradictory results. For example, a multicenter, randomized, double blind, placebo-controlled study on 16 subjects concluded that GDNF administered by i.c.v. injection was biologically active as evidenced by the spectrum of adverse effects encountered in this study [63]. However, GDNF did not improve parkinsonism, possibly because the protein did not reach the target tissue - substantia nigra pars compacta. Likewise, a clinical trial of i.c.v enzyme replacement therapy for central

lysosome storage disease in Tay-Sachs patients also failed [58]. No improvement was observed in patients receiving i.c.v. β-hexaminidase, an enzyme that depletes lysosome storage of GM2 ganglioside [58].

From the delivery standpoint a key challenge for the i.c.v. route is the ependymal lining, which albeit is less restrictive than the BBB still acts as a significant barrier at the ventricle surface hindering the diffusion of substances from CSF into brain parenchyma [122]. Indeed, the brain section of animals receiving i.c.v infusion of basic FGF (bFGF) and BDNF both confirmed that the compounds were distributed only at the ventricle surface with minimal amounts detected in deep brain parenchyma [123–125]. The limited brain uptake following i.c.v. administration could be further compounded by a rapid turnover of therapeutic agents from CSF to systemic circulation, their degradation in ECS, their slow diffusion within brain interstitial fluid and their sequestration by brain tissues (e.g. ependymal, pial and glial cells) [125]. Based on the experience with i.c.v. administration of native forms of proteins one could suggest that incorporating proteins and other therapeutic molecules in suitable delivery systems is perhaps a necessity for future development of drugs using this route. An optimal delivery system would need to display permeability at the ependymal layer, efficient diffusion in brain interstitial fluid and improve bioavailability of the delivered agent within the CSF.

### 4.3 Intraparenchymal injection and implantation

Proteins can be directly administered into brain parenchyma via intraparenchymal injection or implantation. This invasive central route allows bypassing both the BBB and the ependyma lining barrier at the ventricular surface. However, due to limited diffusion in brain interstitial fluid biotherapeutic molecules often locally spread in an area not more than about 2 mm from the site of intraparenchymal injection [123, 126]. The majority of injected substance was then eliminated from the CNS interstitial fluid [127]. For more than a decade, convection-enhanced delivery (CED) has been used to improve the locoregional concentration of substances within brain interstitium by stereotactically placing catheters to deliver a bulk flow upon gradient pressure. The detailed evolution of this technology and the main issues that need be addressed for its further successful development are reviewed elsewhere [128–130]. Although initial animal studies showed that CED of transferrin in brain white matter produced a homogenous penetration in gray matter after 24 hr. infusion [128], CED of protein therapeutics in clinical trials has not been encouraging in most cases. CED of recombinant human GDNF failed to confer clinical benefit to a trial involving 34 PD patients [64]. In this trial GDNF (called "liatermin") was continuously infused directly in the putamen (ipu). The failure of this trial, as suggested by studies of CED of GDNF in primates, might have been related to the extremely high concentration of GDNF around the catheter tip and limited diffusion into surrounding brain parenchyma which resulted in a very limited drug bioavailability [65, 131]. The inconsistent results of clinical studies had decreased enthusiasm about using GNDF for PD treatment with no new trials being reported for several years. However, recently British scientists developed a brain implant device that allows GDNF be given more reliably in the putamen area of the brain. Recruitment for the clinical trial in PD patients using this delivery strategy for GDNF is currently open (UKCRN ID 12085). An early clinical trial involving CED of antibody against EGFR to

malignant glioblastoma patients was not completed because of severe inflammatory reaction prior to tumor debulking [132]. Interestingly, CED of enzyme replacement therapy showed some consistency between preclinical study and clinical trial and appeared to be more successful. Thus CED of glucocerebrosidase to rat and primate brains showed wide enzyme distribution in various brain regions [133]. The CED of this enzyme to neuronopathic Gaucher's patient followed by i.v. enzyme therapy prevented the deterioration in seizures and cranial nerve deficits while showing no clinical signs of toxicity [133]. The variations in the outcomes of CED clinical trials seem to suggest that the slow diffusion still might be a barrier for certain molecules. Moreover, one should carefully examine the surgery or drug associated toxicity arising from this invasive regimen. Nevertheless, radiolabeled antibody [124]I-8H9 is currently undergoing clinical trials for treatment of brain cancers using CED [68, 69]. This is the first time that CED of therapeutic protein targets into the tumor of brain stem aiming to treat patients with diffuse intrinsic pontine glioma. In parallel, there has been a major effort in developing polymer-based nanoparticles and matrices (e.g. liposome, dendrimer, polystyrene nanospheres, maghemite nanoparticles and other lipid nanoparticles) that can be used as implants to produce a sustained release of a substance in localized areas within the brain interstitium [134]. Most of these studies, however, focus on development of small molecule drugs in preclinical or clinical study for treatment or diagnosis of brain tumors.

### 4.4 Intranasal administration

Different from traditional nasal spray that is administered in the vicinity of the turbinates and thus distributes through the systemic circulation, administration at the level of nasal cribriform plate allows substances to bypass the BBB and directly enter the brain with minimum serum exposure. This administration procedure, known as intranasal-to-brain (INB), is more patient friendly compared to other approaches and importantly allows to avoid the BBB, serum clearance and peripheral metabolism. The INB administered proteins have reduced systemic side effects and display rapid central action onset. The olfactory nerve originates in the olfactory mucosa in the anterosuperior nasal cavity and then travels down the olfactory tract until it reaches the olfactory bulb, where the fascicles of the olfactory nerve pass through the cribriform plate, a perforated bone in the base of the skull, into the highest reaches of the nasal cavity. Three known pathways conduct substances across the cribriform plate into the CNS [135]: i) diffusion through the CSF and brain interstitial fluid; ii) retrograde transmission via the olfactory nerves to the olfactory bulb; and iii) retrograde transmission via the trigeminal nerve to the trigeminal nuclei. Growing preclinical evidence suggests that various protein molecules including neurotrophic factors, hormones, antibody fragments, and peptides can reach the brain via INB route in adequate amounts to be effective in the animal models [136–139]. Moreover, several intranasal therapeutics tested in clinical trials (e.g., cholecystokinin [140], corticotrophin-releasing hormone [141] and insulin [142]) elicited measurable central responses. For example, nasal insulin has profoundly improved memory in AD patients [56]. Small peptides, such as antidiuretic hormone arginine-vasopressin (1.1 kDa) and neurohypophysial hormone oxytocin (1 kDa) have shown significant promise as nasal spray products [143–149]. Further, intranasal arginine-vasopressin was detected in human lumbar CSF and shown to increase brain electrical response to various stimuli [150, 151].

At the same time, there are conflicting reports about the advantage of INB route over traditional routes (i.v., s.c. and i.m.) in efficiency of delivering polypeptides to the brain. Clinical trial of intranasal peptide $YY_{3-36}$ (PYY) dosed at 600 μg for 12 weeks failed to induce weight loss in obese patients and furthermore, significant nausea and vomiting were observed in some cases [57]. In animal studies comparable and low levels of brain accumulation were reported after INB and i.v. administration of an agonist of the glucagon-like peptide 1 (GLP-1) receptor, exendin-4 [152]. Therefore, to attain brain concentrations within the therapeutic window, multiple i.n. dosing of therapeutic peptides is required. For example, daily intranasal administration over 10 days of a 16-kDa adipose-derived hormone, leptin reduced the body weight of diet-induced obese mice [153]. It should be also noted that the brain region distribution patterns after i.n. administration vary from substance to substance, suggesting that there might be different mechanisms and pathways involved in the delivery of these substances. In some cases, the INB administered drug is taken up in a brain region irrelevant to the biological target of this drug. For example, interferon β-1b after intranasal administration in rats accumulated in both in CNS as well cervical lymph nodes [154]. Accordingly, there is a need to focus on brain regional distribution of INB-administered therapeutic agents and analyzing their pharmacodynamic responses. In some cases, there might be a possibility to control the brain regional distribution of peptides after their INB delivery. For example, pituitary adenylate cyclase activating polypeptide (PACAP) after i.n. administration in mice exhibited the highest uptake in the striatum and occipital cortex, but combining this polypeptide with β-cyclodextrin increased its uptake in hypothalamus by 8-fold and hippocampus by 2-fold [155].

The unique factors hindering the brain bioavailability of substances administered via the INB route are likely to include the presence of the nasal epithelium barrier, fast clearance of solutes in CSF, and limited penetration at the neuronal pathways. Overcoming the barriers behind nasal epithelium and targeting the delivery-relevant brain regions is of great importance for developing nasal protein therapeutics. Currently most therapeutic agents administered via INB route are formulated with excipients that act either as permeation enhancers at the olfactory epithelium or mucoadhesives to extend residence time in the nasal cavity. In some cases, vasoconstrictors are also co-administered to limit the absorption of the therapeutics by non-olfactory epithelium into the systemic circulation. However, the use of drug delivery systems to improve CNS delivery of proteins after INB administration is relatively poorly explored. Nevertheless, some studies have shown that delivery systems could play significant role in this route. For example, the intranasal delivery of albumin to brain was shown to increase after albumin encapsulation in liposomes [156]. Another example is HRP, a hydrophilic and BBB impermeable enzyme, that most likely crosses the cribriform plate by leaking through the intercellular space of nasal epithelium and then distributes within the brain by diffusion through CSF [116]. Modification of HRP by wheat germ agglutinin has increased the HRP adsorptive endocytosis at both nasal epithelium and olfactory neurons and therefore the trafficking of modified HRP to brain regions is likely to involve the olfactory and trigeminal nerve pathways [157, 158]. Our unpublished data also showed that modification of leptin with amphiphilic block copolymers increased overall brain uptake of intranasal leptin and importantly improved leptin targeting to hypothalamus, the site where leptin controls appetite. Overall, there is a potential of improving CNS

delivery of INB administered protein therapeutics by using drug delivery strategies including these discussed below. The unmet need to treat CNS related disorders hopefully will push forward the studies in this direction.

### 4.5 Intrathecal administration

Therapeutic agents can be injected into the subarachnoid space of the spinal cord allowing them to bypass the BBB and access the brain. This approach known as i.t. administration goes back to the pioneering work of J. Corning who injected cocaine in the lower lumbar vertebrae for the purpose of local spinal anesthesia [159]. Subsequently, it was established as a clinical procedure for administration of drugs with main indications for anesthesia and pain management. Its least invasive and clinically feasible modes include 1) injecting a substance at the lumbar region through lumbar puncture to evoke an acute postoperative response or 2) using a slow infusion device for maintenance of a sustained effect required for chronic management. I.t. administration of protein therapeutics for treatment of CNS related disorders was also explored both in pre-clinical studies and clinical studies. For instance, an antibody against Fas ligand was administered i.t. to treat the acute phase of multiple sclerosis in a mouse model of experimental autoimmune encephalomyelitis (EAE). Notably, this antibody was more potent in blocking Fas-mediated tissue injury and suppressing EAE progression after i.t. administration (20 μg/day for 3 days) than when it was given i.p. (200 μg/day for 3 days) [160]. Another study, however, reported a negligible effect of i.t. infusion of NGF (125 ng/h for 7 days) in a rat model of neuropathic pain [161]. According to a more recent report a 20 kDa glycoprotein, sonic hedgehog (Shh) exhibited neuroprotective effect after i.t. administration in a rat model of ischemic stroke at a dose of 150 μg [162]. More recently, using position emission tomography (PET) five more proteins were shown to rapidly distribute to brain parenchyma after i.t. administration in animal models [61, 163]. Clinical studies reported include BDNF given i.t. to amyotrophic lateral sclerosis patients at five different dose levels starting from 25 μg/day for 12 weeks [164]. Although no efficacy was proven in this study, a notable amount of BDNF was detected in the CSF of patients between day 11 and week 24 of treatment even at the lowest dose applied [164].

As of today, perhaps the most successful example of i.t. application of protein therapeutics for CNS related disorders involves ERT for lysosome storage diseases [61, 165–168]. For example, α-L-iduronidase (rhIDU), an enzyme deficient in mucopolysaccharidosis I (MPS I) was i.t. administered in MPS I model in dogs [168]. The deep penetration of the enzyme into brain regions was shown. Moreover, four weekly i.t. doses of ~1 mg rhIDU produced profound amelioration of the MPS I symptoms. Another study further supported the use of i.t. route over i.c.v. or i.v. routes using I2S, an enzyme deficient in MPS II lysosome storage disease [61]. Intralumbar injection of I2S in monkeys produced nearly same levels of the enzyme in the brain gray matter 4 hr. after treatment as did the i.c.v. injection and over 100-time higher levels than those observed after i.v. administration of this enzyme [61]. Moreover, i.t. I2S treatment has effectively reversed the brain pathology after 3 injections at a dose of 250 μg in a mouse model of MPS II [61]. These efforts have provided momentum towards the clinical use of i.t. I2S in the treatment of MPS II disorder [60].

The physiology and mechanisms underlying the i.t. delivery of macromolecules to the CNS have been discussed elsewhere [169]. Briefly, the transport of solutes from the site of i.t. injection to the brain parenchyma includes 1) initial spread and diffusion in the CSF that is likely driven by pulsatile remixing; 2) clearance through drainage of the CSF; 3) active and pulsation-assisted translocation of remaining substance into the perivascular space; and 4) transfer to brain parenchyma. The latter may involve various mechanisms including penetration at the glia limitans and pial cells, convective transport ("bulk flow"), and anterograde axonal transport. Current data suggest that the location and volume of the administered bolus are the most important factors in the initial spread of therapeutic substance in the CSF after i.t. administration. The subsequent processes, however, are less understood. Although very little is known about immunogenicity of i.t. proteins, one study reported a dose-dependent immune response and a meningeal lymphocytic infiltrate in the dogs that received i.t. administration of rhIDU [168].

Little work was done using drug delivery systems to improve the outcomes of the i.t. administration of therapeutic agents. However, one can expect that nanomedicine strategies can address some common problems of protein therapeutics delivery using this route, such as poor stability of proteins in the CSF and perivascular space, limited permeability of proteins from the perivascular space into the brain and protein immunogenicity. For example, PEGylation of IL-10 increased the CSF concentration of this protein as well as the level and duration of the therapeutic response after i.t. administration of this protein in an animal model of neuropathic pain [170]. Additionally, a use of delivery systems might be worth exploring to improve permeation of i.t. administered proteins.

Overall, the choice of the administration routes for the CNS therapeutics is most challenging due to the restricted anatomical access to the CNS, and the complicated CNS environment. There is no doubt that this choice must account for the efficacy, safety, disease stage (chronic and acute) and patient concerns (convenience and cost). Therefore, during development of new CNS therapeutics, in particular, protein therapeutics, selecting the optimal administration route and the delivery strategy specific for this route is essential and accounts for the success, perhaps, no less than identifying the proper therapeutic target.

## 5. Chemical modification of proteins for CNS delivery

To date some of the most extensive studies to increase protein permeability at the BBB have involved protein chemical modification with various strategies such as a) cationization, b) fusion with cell-penetrating peptides (CPPs), c) fatty acid acylation, d) conjugation with brain targeting ligands, and e) modification with polymers (Figure 3). Notably, the protein modification points, linkers, modification degree and the conjugation chemistry are all important design considerations having a dramatic impact on the properties of resulting conjugates and their *in vivo* performance. Generally, lysine residues of a protein serve as common modification points. Other site-specific chemistries involve protein N- and C-terminus modifications and disulfide bridge insertion [171–180]. Modification of a protein with CPPs and brain targeting ligands can be also accomplished by genetic engineering. The linker in such fusion proteins need be designed in such a way that it allows the independent folding of each protein and also enables release of the two separate proteins if needed.

However, detailed consideration of these design factors is outside of the scope of this review. Below we present the different chemical modification strategies and assess their state of development and promise for future pharmaceutical use. The representative examples of these strategies and some principal observations are presented in Table 2.

### 5.1 Cationization

A simplest way to cationize a protein is to chemically modify its carboxylic acid groups with synthetic (e.g. hexamethylenediamine) or natural amines (e.g. putrescine, spermidine and spermine). In an early study Pardridge and co-workers modified native albumin (pI 4) with hexamethylenediamine and produced a cationized albumin (pI > 8). They demonstrated that β-endorphin, a BBB impermeable peptide, after conjugation with such cationized albumin was rapidly taken up by isolated brain capillaries *in vitro* in a temperature dependent manner. Moreover, the autoradiography data showed that the conjugate crossed the capillaries and distributed in the brain parenchyma following intracarotid injection in rats [181, 182]. An increase in the brain uptake was also observed for cationized IgG (an increase in pI from 5–6 to >10) in both *in vitro* and *in vivo* [183]. Both studies reported that the uptake of cationized proteins in the brain was saturable. Even though the assessment of BBB function was not a part of this study, authors claimed that the increase in brain uptake was not related to the breakdown of the BBB [183]. Subsequently, Poduslo and colleagues demonstrated ability of several cationized proteins (e.g. superoxide dismutase (SOD), insulin, albumin, IgG and neurotrophic factors) to cross the BBB without disrupting its integrity [184, 185]. In these studies the permeability of the cationized proteins at the BBB after i.v. administration was assessed by calculating the permeability coefficient times surface area (PS), a reliable PK measure of brain entry. Moreover, the lack of the BBB leakiness was unequivocally proven by little or no change in the volume distribution of a native protein co-injected with the cationized protein [184, 185].

Some therapeutic or disease relevant effects of cationized proteins in animal models were also reported. For example, systemic administration of a putrescine-modified SOD resulted in neuroprotective effects in rats with global cerebral ischemia [186]. The diamine- and gadolinium-derivative of human Aβ peptide was shown to have enhanced *in vitro* binding to AD amyloid plaques and increased *in vivo* permeability at the BBB of normal adult mice. Specific targeting of the modified Aβ peptide to amyloid plaques in the brain was also demonstrated in a transgenic mouse model of AD [187].

It was suggested that cationization increases permeability at the BBB by promoting interaction and transcytosis of the protein across the BMECs. However, the exact mechanisms remained unclear since permeability did not directly correlate with the number of positive charges of the polyamines in the cationized protein [184, 188]. Along with an increased BBB permeability cationization also resulted in undesirable consequences such as an increase in the serum clearance of the protein. For example, when was modified by polyamine, its plasma half-life time dramatically decreased from 3 min to about 0.6 min [184]. Similar effects were observed for cationized IgG, albumin and insulin [184]. The decreased half-life could offset improved BBB permeability of the cationized proteins and decrease their net brain accumulation especially for proteins having intrinsically rapid blood

clearance. Moreover, despite that cationization was not reported to disrupt BBB in these studies, concerns about dose-limited toxicity of cationic substances persist. In particular, although low doses of cationized IgG used in PK studies ware safe for peripheral organs and brain capillaries [183], considerable toxicities (immune complex formation, membranous nephropathy) were observed after injecting therapeutically relevant doses to rabbits [189, 190]. Likewise, administration of high dose of protamine alone also resulted in an increased cerebral and peripheral vascular permeability [191–194]. Thus potential toxicity of cationized proteins and the resulting limitations to the therapeutic window of possible therapeutic agents are the main factors that have limited the development of this technology for the clinical use.

## 5.2 Protein fusion with CPPs

Modification with CPPs is yet another approach to improve brain delivery. Examples of CPPs derived from natural proteins are trans-activating transcriptional activator (TAT), penetratin, and the Syn-B vectors. Other CPPs homoarginine vectors, the model amphipathic peptide, transportan and chimeric peptides (sequence signal-based peptide and fusion sequence-based peptide) are engineered artificially. Heterogeneous in size (10–27 amino acids) and sequence, all CPPs are comprised of basic amino acids and are cationic. Translocation of CPPs through cell membrane may occur by passive diffusion. Alternatively, CPPs can destabilize the phospholipid bilayer and form inverted micelles that enable entry of CPP and its attachment, e.g. proteins into cells without leaving an aqueous environment [195–198]. More detailed description of the CPP internalization mechanisms, and other properties such as stability, toxicity and immunogenicity were reviewed elsewhere [199]. Here we focus on use of CPPs for delivery of proteins to CNS.

Schwarze and colleagues published a seminal work demonstrating ability of CPP to deliver proteins across BBB [200]. In their study the $NH_2$-terminal TAT (47–57)-galactosidase fusion protein (120 kDa) injected i.p. in mice was detected by immunochemical staining initially at 2 hr in brain microvessels and then at 4–8 hr in brain parenchyma. No PK studies were performed. Nevertheless galactosidase activity was visualized in sagittal and coronal brain sections as well as in liver, kidney, lung and heart (myocardium) and spleen. TAT did not appear to disrupt BBB as the Evan's blue albumin complexes co-injected with TAT were excluded from the brain tissues. Subsequently, TAT peptide was fused with GDNF and injected i.p. in a mouse model of PD. The fusion protein crossed the BBB and reached substantia nigra as was shown by immunohistochemical staining. However, the treatment did not prevent the loss of dopaminergic neurons in PD mice, possibly because the amount of the fusion protein delivered to the target site was not sufficient [201]. A TAT-based system was also used to deliver Bcl-xL protein, a well-characterized death-suppression molecule, to the CNS for treatment of stroke. Intraperitoneal injection of TAT and Bcl-xL fusion protein resulted in a robust protein transduction in neurons, and a dose-dependent decrease of cerebral infarction in a mouse middle cerebral artery occlusion (MCAO) model of ischemic stroke [202]. Similarly, a reduced infarct volume and neurological deficits were observed after i.v. injection of TAT-Bcl-xL fusion protein 1 hr. before or immediately after the ischemia induced in a rat MCAO model [203]. A recent study reported that TAT-leptin fusion protein was i.v. injected to mice fed with high-fat diet. Immunohistochemical staining

suggested increase in leptin accumulation in hypothalamus in the TAT-leptin treated mice, compared to the unmodified leptin or saline-treated animals. Importantly, TAT-leptin also prevented body-weight gain more efficiently compared to leptin [204]. Cai et al. recently described positive effects of TAT-mediated delivery of neuroglobin (Ngb) on focal cerebral ischemia outcome in mice [205]. After i.v. injection the TAT-Ngb fusion protein was detected in mice brain tissues by immunohistochemistry and western blotting. The group treated with TAT-Ngb 2 hr. before MCAO showed smaller brain infarct volume and improved neurologic outcomes compared to the control groups. Moreover, the group treated with TAT-Ngb after MCAO and reperfusion showed significantly increased neuronal survival in the striatum, compared to the controls [205].

Besides TAT some other CPPs, such as Syn-B vectors and Rabies virus glycoprote-inderived peptide (RDP), were also shown to deliver small molecules and proteins across BBB [206, 207]. For example, Xiang et al reported efficient hippocampus targeting by a β - galactosidase-RDP fusion protein [206]. Interestingly, a simple mixing of a protein with CPP also improved delivery of multiple proteins such as β-galactosidase, human IgG and IgM to mouse brain [208].

However, CPP have displayed various toxicities including neurotoxicity, endothelial cell apoptosis and inflammation [199], which decreased likelihood of their translation to clinic use. Another obstacle to future product development is a non-specific penetration of CPP-modified proteins into peripheral tissues. Thus a case-by-case preclinical toxicology study accounting for stability, efficacy and safety must be performed to evaluate further possibilities of using this technology for specific CNS therapeutic application.

## 5.3 Fatty acid acylation

Early work by Chekhonin and Kabanov described protein modification with fatty acids for brain delivery [209]. For example, a neuroleptic drug (trifluoperazine) was attached to Fab-fragments of antibodies against gliofibrillar acid protein (GFAP) or brain specific α2-glycoprotein (α2-GP). The drug-Fab conjugates were then modified with stearate in reverse micelle system formed by a surfactant, sodium bis-(2-ethylhexyl)sulfosucciate (Aerosol OT) in octane. Stearoylated Fab fragments of brain-specific antibody exhibited brain accumulation and a drastic increase in neuroleptic activity of trifluoperazine following intracoratid injection into rats. In contrast, fatty acylated Fab fragments of nonspecific antibodies accumulated in the liver rather in the brain [209]. Subsequent studies using BMECs as an *in vitro* BBB model demonstrated that stearoylation of ribonuclease A increased the transport of this enzyme across the BBB by almost 9-fold [210]. In another study Slepnev and colleagues used a membrane-impermeable enzyme, HRP as a model protein to examine effects of stearoylation of the protein on its interaction with cells [211]. This work demonstrated that stearoylation increased binding and internalization of HRP in mammalian cells, albeit the internalized protein accumulated in endocytic vesicles but not in the cytoplasm [211]. Notably, the stearoylated HRP displayed much greater binding with a hepatic cell line than with epithelial cells, which could be due to the presence of the fatty acid binding receptor in hepatocytes. Subsequent PK study from Kabanov and Banks' laboratory demonstrated that after i.v. injection stearoylated HRP was able to cross mouse

BBB at a higher influx rate than the native HRP [212]. This work also reported about 13% increases in brain uptake of stearoylated HRP over 200 min as compared to native HRP. The volume of distribution of fatty acylated HRP also increased due to its non-specific distribution in liver and other organs [212]. Shen and colleagues reported that palmitoyl residue conjugation through a disulfide linker to interferon α enhanced its circulation and liver accumulation; the effect of palmitoylation on brain uptake of interferon α was not reported [213].

Overall fatty acylation is likely to result in the increased binding of proteins to brain microvessel endothelial cell membranes through hydrophobic interactions of the attached lipid anchor with the membrane bilayer [212]. In addition many other factors can contribute to delivery of proteins following lipidization. Cellular binding might be further increased when the modified protein itself contains a polybasic motif which in addition to lipid carrier serves an anchor for interaction with cell membrane [214]. A transporter-mediated mechanism might come in play when proteins are modified with essential fatty acids, such as linoleic acid, which is naturally transferred to the brain by transporters [215, 216]. Other factors including plasma protein binding, intracellular sequestration, non-target organ uptake can contribute to plasma PK and biodistribution and should be taken into account when developing CNS targeted therapeutics using this approach.

As of today perhaps the most advanced application of fatty acylated proteins is the development of long-acting anti-obese hormones that originate from the gut-intestine tissue and act partially or exclusively within the CNS to control appetite and energy consumption. Interestingly, fatty acylation of gut-brain hormones is a naturally occurring phenomenon. In fact, the octanoic acid acylated ghrelin acts as an important cognate ligand to stimulate growth hormone release and regulate energy hemostasis [217]. Post-translational O-n-octanoylation of ghrelin at the serine 3 position is indispensible to the hormone binding to and activation of the growth-hormone secretagogue receptor [218, 219]. The brain PK study did show that octanoylation of ghrelin has a dramatic effect on its transport characteristics across the BBB [220]. Another example is liraglutide, a GLP-1 analog modified with a C16 fatty acid chain that is currently being tested in Phase III clinical trial as an anti-obesity drug [221–224]. This modified peptide analog displays notable improvement in its PK profile (longer circulation, smaller volume of distribution), and can be used once a day to replace exenatide (a native form of GLP-1) given twice daily. Interestingly, GLP-1 as well as many other gut-brain hormones control appetite and thermogenesis at two sites: peripherally by signaling the vagus nerve surrounding gut-intestine and centrally acting at the receptor in the brain. However, in spite of improvement in the hormone's peripheral circulation, no brain PK data were reported to support the role of fatty acylation to increase GLP-1 brain uptake. Further discussion of the application of the fatty acylation for development of gut-brain hormone therapeutics can be found elsewhere [225].

## 5.4 Conjugation with brain-targeting ligands

To maintain homeostatic environment of CNS brain endothelial cells express a variety of receptors and transporters that mediate blood-to-brain transcytosis of hormones, transport proteins and other essential substances including insulin, growth factors, low-density

lipoprotein (LDL), amino acids, and glucose. Low molecular mass drugs or polypeptides can be designed to closely resemble endogenous ligands of these receptors. Conjugation of such artificial ligands to polypeptides (as well as other biomacromolecules) can improve delivery of these molecules to the brain [226–228]. Such brain delivery strategy is frequently referred to as "Trojan horse". This strategy has a few well-known caveats. First, the ligand-modified molecules compete with the endogenous ligands, which can potentially lead to 1) ineffective brain targeting; or 2) side effects induced by endogenous ligand deficiency in the brain. Second, chemical conjugation can affect binding affinity of the ligand to its receptor and/or the biological activity of molecule to be delivered. The linkers used for conjugation can significantly alter the PK profile of the conjugate and affect the release and stability of delivered molecules. Below we consider several such Trojan horse approaches using various targeting ligands such as antibodies against transferrin receptor (Ab-TfR), antibodies against insulin receptor (Ab-InsR), ligands of LDL receptor-related proteins 1 and 2 (LRP1 and LRP2), melanotransferrin (MTf), receptor associated protein (RAP) and angiopep.

**Ab-TfR**—TfR is responsible for iron transport to the brain. Both transferrin and Ab-TfR bind with TfR in brain endothelium albeit they use different binding sites. However, transferrin is not a good candidate for the Trojan horse strategy because its TfR binding site is already saturated by endogenous transferrin. Pardridge and colleagues reported that Ab-TfR (OX26), a mouse IgG2a monoclonal antibody against rat TfR accumulated in the brain to a greater extent than in liver or other organs [229] and can serve as vehicle for delivery of various proteins to the brain [230]. Thus, basic bFGF conjugated to OX26 through biotin-streptavidin reaction accumulated in rat brain at 0.05% injected dose/g brain tissue, which was 5-fold higher than non-conjugated bFGF [231]. A single i.v. injection of a bFGF-OX26 conjugate produced nearly 80% reduction in infarct volume in a rat model of transient ischemic stroke [232]. In another study conjugation of OX26 to EGF resulted in increased the brain uptake and accumulation of EGF in brain tumors after i.v. administration [233]. Other proteins such as vasoactive intestinal peptide (VIP), BDNF as well as nucleic acids were also conjugated with OX26 for brain delivery [234, 235]. An alternative to OX26, a chimeric monoclonal antibody against mouse TfR (CTfRMAb) was also used in production of fusion proteins for brain delivery of erythropoietin (EPO), α-L-iduronidase (IDUA), anti-Aβ amyloid antibody, Aβ$_{1–40}$ peptide, GDNF, tumor necrosis factor decoy receptor (TNFR) and others [236–246]. Therapeutic effects of the fusion proteins in relevant CNS disease models were also demonstrated. For example, Boado et al. reported that IDUA-CTfRMAb fusion protein reduced brain intracellular lysosomal inclusion bodies in a mouse model of MPS I [239]. Studies were also reported using antibody against human TfR. These antibodies may have greater potential for clinical translation than OX26 and CTfRMAb, which are specific to mouse and rat, respectively. For example, Xu et al. used scFv fragment of antibody against human TfR to target cationic immunolipoplex to breast tumor [247]. Another study demonstrated brain accumulation of the scFv fragment against human TfR in mice [248].

The transcellular permeability at the BBB is governed by the internalization of the molecules at the luminal side of the brain endothelium, the intracellular sorting of the molecules in the endothelial cells and the release of these molecules from the abluminal side

of the endothelium. In spite of the aforementioned reports showing that Ab-TfR improved the brain delivery of various cargo the use of this strategy for the delivery proteins across the BBB to brain parenchyma was a matter of discussion for over a decade. In early days Moos and Gosk have shown that most of brain-associated OX26 accumulated in brain capillary endothelium and not in brain parenchyma [249, 250]. It was not until recently when thorough studies by scientists from Genentech, Inc have provided an insightful understanding of this issue [117, 251, 252]. They confirmed by microautoradiography and confocal microscopy that the majority of i.v. Ab-TfR was indeed entrapped in the brain endothelium due to lysosomal sorting. To improve BBB penetration a lower affinity antibody was developed and evidenced by less degradation within the brain endothelium and maintains the capability to bind with TfR and internalize at the brain endothelial luminal side but also is easily released at the abluminal side of the BBB. They showed that at therapeutically relevant concentrations in mouse, this lower affinity antibody was released from the BBB and broadly distributed in brain parenchyma. The brain delivery of this low affinity Ab-TfR was then significantly improved after i.v. administration [117]. This example underscores the importance of in-depth understanding of intracellular trafficking (e.g. lysosome escape, early endosome targeting) and sorting of the delivered materials within the brain endothelium, a field that has been insufficiently explored.

There is also a possibility to avoid covalent conjugation of Ab-TfR and the transported molecules, which can facilitate pharmaceutical development of the respective delivery systems. Taking advantage of the inherent Y-shape structure of antibodies, Genentech scientists also made substantial progress by engineering a bi-specific antibody with one Fab fragment (arm) derived from low affinity Ab-TfR and another Fab fragment (arm) containing an antibody against β-secretase (Ab-BACE1) [117, 252]. Without additional changes in its molecular weight, size and overall structure, this bi-specific antibody embedded therapeutic function of Ab-BACE1 for the treatment of AD and transcytosis capability at the BBB arising from the low affinity Ab-TfR. Indeed, the bi-specific antibody accumulated in the brain in a greater amount than Ab-BACE1 and significantly reduced the brain Aβ levels in a mouse model of AD [117, 252]. Unfortunately, targeting the transferrin receptor at the BBB apparently also increases the peripheral exposure of the bi-specific antibodies, which raises some safety concerns. It was shown that Ab-TfR after i.v. injection in mice at doses starting from 1 mg/kg caused acute clinical signs and decreased reticulocyte count [252]. Therefore, prospects of the clinical use of Ab-TfR containing bi-specific antibodies remain uncertain.

**Ab-InsR—**A high affinity insulin receptor (InsR) in brain endothelium binds insulin and enables its transport across the BBB. Insulin cannot be used as a carrier protein *in vivo* due to a risk of hyperglycemia. However, Pardridge and colleagues have successfully used Ab-InsR to deliver proteins to the brain [253]. In particular, a conjugate of GDNF with fully humanized antibody against human InsR (HInsR) exhibited neuroprotective effect in a rat model of transient ischemic stroke [254, 255]. This conjugate was also shown to accumulate in a rhesus monkey brain. The fusion constructs comprising monoclonal antibody against HInsR with EPO, TNFR and anti-Aβ amyloid ScFv were also evaluated as potential therapeutic agents [256–259]. However, concerns about possible interference of such

constructs with insulin receptor and adverse effects on glucose metabolism decrease enthusiasm about their possible clinical use.

**LRP1 and LRP2 Ligand**—LRP1 (CD91) and LRP2 (gp330) are type I transmembrane proteins that belong LDL receptor superfamily. LRP1 and LRP2 both contain intracellular and transmembrane domain along with an extracellular domain characteristic of LDL receptor family [260, 261]. Both proteins interact with a wide range of ligands and facilitate their endocytosis. LRP1 also plays a major role in regulation of signaling pathways. The dysfunction of LRP1 has been associated with a number of CNS related disorders, including AD, stroke and multiple sclerosis [262–264]. A large number of ligands are shared between LRP1 and LRP2, such as lipoprotein lipase, α2-macroglobulin, receptor associated protein (RAP), lactoferrin, tissue- and urokinase-type plasminogen activator (tPA/uPA), etc. However, some ligands (e.g. melanotransferrin (or P97), Aβ precursor protein, complement C3, apolipoprotein E, and HIV-1 Tat protein) exhibit relatively higher specificity to LRP1, whereas others (e.g., apolipoprotein J, Aβ bound to apolipoprotein J and apolipoprotein E, very-low-density lipoprotein (VLDL)) are more specific to LRP2 [265–271]. Some of these ligands, such as angiopep, MTf and RAP, have shown capability for LRP mediated transport across the BBB and were used for delivery of proteins to the brain.

**Angiopep**—Angiopep is a family of 19 amino acid sequences derived from aprotinin, a 6.5 kDa pancreatic trypsin inhibitor that by itself is rapidly transported across the BBB through LRP mediated pathway [272]. One member of this family, angiopep-2, displays the highest transcytosis rate at the BBB and best accumulation in brain parenchyma among all identified angiopep peptides [272, 273]. It is now clearly shown that angiopep-2 is transported through LRP1 mediated pathway [274, 275]. Currently the most successful application of Angiopep in CNS delivery is ANG1005 (Angichem, Inc), a paclitaxel-angiopep-2 conjugate that contains three paclitaxel molecules attached to two lysine residues and N-terminus of angiopep-2. ANG1005 was shown to bypass Pgp and exhibit greater brain uptake than free paclitaxel in *in situ* mouse brain perfusion study [276]. ANG1005 has shown some antitumor effects in intracerebral glioblastoma in mice, although no direct comparison with paclitaxel was made [276]. Nevertheless the drug was evaluated in the phase I clinical trial in the patients with recurrent malignant gliomas and brain metastases and was fairly well tolerated showing a toxicity profile similar to that of Taxol. Importantly, the concentration of paclitaxel in tumors removed from some ANG1005 treated patients exceeded that previously reported for patients treated with Taxol [277, 278]. This might suggest that the drug penetration at the BBB using ANG1005 was improved compared to regular Taxol chemotherapy. Two other angiopep-2-based conjugates, ANG1007 (angiopep-2-doxorubicin) and ANG1009 (angiopep-2-dimethylglycine etoposide), also demonstrate improvement in brain accumulation and brain tumor inhibition in animal studies [279]. The use of angiopep-2 for the brain delivery of various macromolecular species was also reported. Examples include polyamidoamine dendrimers-PEG/DNA nanoparticles [280], PEG-polylactic acid (PLA) polymeric micelles [281], poly(ethyleneimine)-PEG (PEI-PEG) based polymeric micelles loaded with amphotericin B [282], and a few others [283–286]. Angiochem also reported conjugation of angiopep-2 to anti-HER2 antibody (ANG4043) and anti-EGFR antibody [287, 288]. In both cases, modification was reported to increase the

antibody transport rate across BBB as shown using *in situ* brain perfusion and fluorescent imaging in a mouse [287, 288]. In another study, angiopep-2 conjugation to a short sequence analgesic peptide, neurotensin (1.6 kDa) (ANG2002) appeared to be successful in targeting thins peptide to the brain for the treatment of chronic pain [289]. At least 10 times increase in the neurotensin transport across the BBB and a significant improvement in the analgesic effect for pain control in three rat models of pain were reported. However, along with these promising reports there were some discouraging results obtained using the angiopep-2 strategy for the brain delivery of a lysosome enzyme, arylsulfatase A (ASA) [290].

**MTf**—MTf, an iron-binding homolog of transferrin was initially recognized as a melanoma-specific marker (also called melanoma tumor antigen P97) [291]. It was later detected in other tissues including brain, where it is expressed by endothelial cells and activated microglia in AD patients. Despite structural similarity to transferrin, MTf exists mainly in membrane-bound form having a C-terminal glycosyl phosphatidylinositol anchor (25 residues) and two homologous extracellular domains (342 and 352 amino-acid residues). A soluble form of MTf (sMTf) was detected in the blood at very low concentration. Interestingly, i.v. injected sMTf accumulated in the brain although to a lesser extent than transferrin [292]. Beliveau's group and others demonstrated that recombinant human MTf passed into the brain across BBB at a high rate via LRP1 receptor-mediated pathway [267, 293]. Owing to a very low level in the blood and fast rate of transcytosis across the BBB, MTf could be used as a carrier for drug delivery to the brain. Indeed, Tang and colleagues engineered a fusion protein of sMTf and cell surface coxsackie-adenovirus receptor (sCAR), which binds as adaptor protein to both adenovirus serotype 5 (Ad5) and brain endothelium receptor and promotes internalization of the Ad5 vector. Using an *in vitro* BBB model, these authors demonstrated that sMTf-sCAR fusion protein redirects the cell trafficking of the Ad5 vector and facilitates its transport across the brain endothelial cells [294]. Moreover, sMTf was able to successfully deliver conjugated doxorubicin molecule to brain tumors *in vivo* [295]. Similarly, sMTf-adriamycin conjugates crossed BBB and prolonged survival of animals bearing intracranial gliomas or peripheral mammary tumors, compared to the animals treated with the same cumulative dose of free-adriamycin [296]. BioMarin Pharmaceuticals Inc. is currently developing sMTf as delivery modality for enzyme replacement therapy in the brain [297].

**RAP**—RAP is a 39 kDa protein located mainly in endoplasmic reticulum where it plays crucial role in assisting folding and trafficking of the LDL receptor family including LRP1 and LRP2. Upon i.v. administration RAP binds LDL receptor family and therefore inhibits clearance of other ligands such as tPA [298, 299]. Pan and colleagues reported that RAP crossed BBB and reached brain parenchyma via LRP2-mediated pathway at a higher rate than transferrin and MTf [300]. Moreover, Prince showed that conjugation of RAP to lysosome enzyme, IDUA and acid α-glucosidase enhanced enzymes intracellular accumulation and substrate depletion within fibroblasts thus suggesting the possibility of RAP use as a drug carrier [301]. However, therapeutic potential of RAP conjugates for treatment of brain-related lysosome storage disease remains untested.

## 5.5 Protein modification with hydrophilic and amphiphilic polymers

Perhaps the most successful approach to improvement of bioavailability of proteins is PEGylation - covalent attachment of PEG polymer chains to protein molecules. Frank Davis and Abraham Abuchowski reported the very first studies on protein PEGylation in 1970s. Using catalase and albumin as model proteins, they discovered that attachment of PEG (1.9 or 5.0 kDa) improved protein circulation and serum stability and reduced immunogenicity [302, 303]. Since then, PEGylation has been widely used to modify proteins and helped to advance development of protein therapeutics tremendously [304]. Various aspects of PEGylation, such as chemistry of PEGylation, analytic and bioanalytic characterization, the PK and pharmacologic properties and the clinical applications are extensively discussed in literature [180, 305–312]. PEGylation of proteins can prolong their blood circulation, increase their serum stability, and reduce their immunogenicity [305, 310, 311, 313]. Peptide agonists of the GLP-1 receptor are rapidly gaining attention as antidiabetic agents, since in addition to increasing glucose-dependent insulin secretion, they also cause weight loss. For example, oxyntomodulin (OXM), a natural peptide with sequence homology to both glucagon and GLP-1, was recently modified with PEG to increase this peptide's half-life and decrease its degradation by dipeptidyl peptidase IV (DPP-IV) [314]. The PEGylated OXM exerted an anti-hyperglycemic effect in diet-induced obese (DIO) mice in a glucose-dependent manner, and thus has shown potential as novel once-weekly GLP-1 mimetic with both glucose-lowering activity and weight loss efficacy. However, albeit PEGylation of leptin increased this hormone's half-life in circulation it did not improve its brain uptake in animals. Moreover, PEGylated leptin failed to induce weight loss in obese patients.[315–317] Therefore it appears that PEGylation is not successful as a brain targeting strategy. This may be explained by increased molecular weight and hydrophilicity of PEGylated proteins, both unfavorable for transport of proteins across cellular barriers. Therefore, albeit PEGylation improves serum bioavailability of a protein and thus increases its exposure to the brain capillaries these effects are offset by reduced permeability of PEGylated proteins across the BBB [318, 319]. In addition to PEG some other hydrophilic polymers, such as natural polysialic acid (PSA) [119], dextrin [320–323], and hyaluronic acid [324] as well as synthetic N-(2-hydroxypropyl)-methacrylamide (HPMA) [325] were also used for protein modification. Most of these protein-polymer conjugates have extended circulation time and improved stability in serum as compared to native proteins. However, modification of proteins with these hydrophilic polymers, like in the case of PEGylation, improves the PK profile of proteins but not their ability to cross the physiological barriers.

The protein serum bioavailability and ability to penetrate across brain endothelium can be improved by modification of proteins with amphiphilc block copolymers [225]. For example, Pluronic block copolymers (also termed "poloxamers") consist of hydrophilic poly(ethylene oxide) (PEO or PEG) and hydrophobic poly(propylene oxide) (PPO or polypropylene glycol (PPG)) segments arranged in a basic A-B-A structure: PEO-PPO-PEO (PEG-PPG-PEG). By changing the lengths of the PEO and PPO segments one can vary the hydrophilic-lipophilic balance of these polymers and alter their ability to interact with each other and lipid membranes. A characteristic of Pluronics is the ability to self-assemble into micelles in aqueous solutions above the critical micelle concentration (CMC). Already a quarter of century ago Pluronic micelles conjugated with antibodies to brain specific

antigens (e.g. α2-GP) were shown to deliver solubilized compounds to the CNS after i.v. administration in mice [326]. Interestingly, selected Pluronics are potent inhibitors of Pgp and increase entry of Pgp-substrates into the brain across BBB [327–329]. These copolymers were shown to cross membranes of BMECs and enter brain tissues in mouse models [330, 331]. Moreover, some copolymers, such as Pluronic P85 were shown to internalize in primary neurons [332]. These copolymers follow cell trafficking itinerary similar that of cholera toxin B, including binding with cholesterol-rich domains in cell membrane and then internalization via caveolae-mediated or caveolae- and clathrin-independent endocytosis [333, 334]. Based on these observations Pluronics were used to modify proteins to deliver them across the BBB. Initially, HRP modified with relatively hydrophobic Pluronic block copolymers (P85, L81 and L121) was shown to cross *in vitro* BBB model and following i.v. administration in mice, HRP-P85 exhibit higher permeability at the BBB than HPR alone and accumulated in brain parenchyma [212, 335]. Subsequently, SOD1 modified with Pluronic P85 or L81 was shown to internalize into neuronal cells, while retaining enzymatic activity and acting as a scavenger of intracellular superoxide induced by angiotensin II [336]. Moreover, after intracarotid administration in rabbits this conjugate also induced a central physiological response by inhibiting angiotensin II-induced increase in the arterial pressure, not observed after native SOD1 injection [337].

Protein modification with Pluronics was used recently for development of an anti-obese drug on the base of leptin [78, 338, 339]. Leptin, a candidate for the treatment of epidemic obesity, has failed in part because of impairment in its transport across the BBB that develops with obesity [340–342]. It was suggested that modification of leptin with Pluronic P85 might permit this protein to penetrate the BBB independently of its transporter, thereby overcoming peripheral leptin resistance. PK studies demonstrated that Pluronic conjugate was transported across BBB at an influx rate similar to native leptin, but via non-saturable mechanism independent of leptin transporter [338]. Importantly, the conjugate reduced food intake following i.c.v. or i.v. administration in healthy mice and in mouse models of obesity (ob/ob, and diet-induced obese mouse) [338, 339]. We further generated two new leptin-P85 conjugates: one, Lep(ss)-P85(L), containing one P85 chain and another, Lep(ss)-P85(H), containing multiple P85 chains. Lep(ss)-P85(L) crosses the BBB using the leptin transporter, and exhibits improved peripheral PK along with increased accumulation in the brain compared to unmodified leptin. Lep(ss)-P85(H) also has improved peripheral PK but in a striking difference to the first conjugate penetrates the BBB independently of the leptin transporter via a non-saturable mechanism. The results demonstrate that leptin analogs can be developed through chemical modification of the native leptin with Pluronic P85 to overcome leptin resistance at the level of the BBB, thus improving the potential for the treatment of obesity [339].

Although the use of Pluronics for brain delivery of proteins is still under investigation, the translation of this technology to a clinic is promising. One advantage of using Pluronics for brain delivery is their relatively low toxicity. Pluronic copolymers are FDA-approved excipients and are widely used in a variety of clinical applications. No CNS related toxicity was reported in Phase I and II clinical trials of doxorubicin formulated with Pluronics ("SP1049C") [343, 344]. Studies show that Pluronics at concentrations substantially

exceeding those used in protein-Pluronic conjugates have little if any toxic effect on brain endothelial cells *in vitro* and *in vivo* and do not alter paracellular permeability of BBB [327, 328, 345].

Another technology showing potential is protein modification with amphiphilc poly(2-oxazoline)s (POx) block copolymers [346]. POx polymers have been explored in various drug delivery and other biomedical applications [347–357]. Their advantages compared to PEG and Pluronics include greater stability, greater synthetic versatility allowing introduction of various functionalities both the polymer repeating units and terminal groups. This makes them promising candidates for protein brain delivery. Hydrophilic POx homopolymers such as poly(2-methyl-2-oxazoline) (PMeOx) and poly(2-ethyl-2-oxazoline) (PEtOx) are considered as alternatives to PEG in applications similar to PEGylation [358, 359]. The amphiphilic POx block copolymers exhibit relatively low cytotoxicity and can transport into cells similarly to Pluronics [356, 357]. We have reported recently that HRP modified with amphiphilic block copolymers of PMeOx and 2-butyl-2-oxazoline (P(MeOx-*b*-BuOx), or PEtOx and 2-butyl-2-oxazoline (P(EtOx-*b*-BuOx) transported into intracellular compartments in both MDCK and Caco-2 cells [360]. Next, we modified SOD1 with two aforementioned POx block copolymers [361]. Similar to SOD1-Pluronic conjugates, SOD1-POx conjugates retained high stability and catalytic activity after modification. Moreover, SOD1-P(EtOx-*b*-BuOx) conjugate showed high uptake level in CATH.a neurons and efficiently scavenged intracellular superoxide induced by Ang II stimulation. This conjugate utilized caveolae-mediated and/or clathrin and caveolae-independent endocytosis for cell entry. After i.v. administration in mice radiolabeled SOD1-P(EtOx-*b*-BuOx) displayed longer blood half-life compared to native SOD1, crossed BBB by non-saturable mechanism, and reached brain parenchyma [361]. Even though this new technology has already shown potential in enhancing delivery of proteins to the brain, a clear understanding of POx and protein-POx interactions with brain endothelium is needed to take the full advantage of the structural versatility of this type of polymer.

## 6. Particle-based carriers for CNS delivery of proteins

Numerous studies have shown that encapsulation of therapeutic proteins in nano- or micron size particles decreases protein immunogenicity and improves protein stability and circulation time (Figure 4). Liposomes and PLGA nanoparticles are possibly the most extensively investigated types of carriers for protein delivery. Other systems investigated in the context of CNS delivery include poly(butylcyanoacrylate) (PBCA) nanoparticles, and more recently, polyion complexes. Some other materials such as PEG-silica, bolaamphiphilies, chitosan, PEG-polylactide (PLA), PEG-poly($\epsilon$-caprolactone) (PCL) and PLA-D-$\alpha$-Tocopheryl polyethylene glycol succinate (TPGS) were also evaluated for brain delivery [283, 371–377]. Unfortunately, such particle-based carriers generally do not cross BBB. Surface modification with specific brain targeting moieties may provide opportunities to enhance brain the delivery of particles but the effectiveness remains questionable [378–380]. Nevertheless interest in particle-based systems for delivery of therapeutic agents to CNS persists due to ongoing efforts in application of these systems with drugs having a peripheral mode of action. Notably, majority of such studies using particle-based carriers involve delivery of low molecular mass therapeutics to the CNS [381, 382], with only

relatively few examples reporting CNS delivery of proteins [383]. Because most carrier-based technologies were originally developed for delivery of low molecular mass drugs, in some cases there is an additional challenge in modifying the carrier technology to facilitate protein formulation, ensure high protein loading and stability. Below we consider some of these carries and their applications for protein delivery to the brain.

## 6.1 Liposomal carriers

Liposomes have been extensively investigated as carriers for delivery of small drugs, proteins, DNA, siRNA and imaging agents [384–387]. Few of these studies involved delivery of proteins to the brain. For example, over 30-years ago it was reported that encapsulation of proteins (β-galactosidase, thyrotrophin-releasing hormone (TRH)) in neutral (phosphatidylcholine (PC), cholesterol (Chol)) or anionic (PC, Chol, dicetylphosphate or phosphatidylserine (PS)) liposomes can increase brain accumulation of these proteins after i.v. administration [388, 389]. Interestingly, TRH loaded in neutral liposomes showed greater brain uptake and physiological effect (rise in body temperature) than TRH in anionic liposomes. Incorporation of TRH in cationic liposomes (PC, Chol and stearylamine) also increased the protein brain uptake. However, stearylamine caused epileptic seizures and cerebral tissue necrosis this and therefore, due to toxicity this formulation was not pursued [389]. Nevertheless, cationic liposomes were further used to deliver SOD1. Several studies demonstrated that SOD1 formulated in cationic liposomes administered i.v. can reduce cerebral infarct volume size in ischemic stroke and brain trauma animal models [390–393]. Though reasons for the improved brain delivery of the liposome-incorporated proteins remained unknown, it was speculated that liposome could cross-lipophilic membranes of brain endothelial cells [389]. To the best of our knowledge no evidence of that has been provided so far to support this mechanism. It was also suggested that cationic liposomes can produce a transient pathologic opening of the BBB during experimental stroke, which can explain at least some of the effects observed [394, 395]. Overall, the translation of cationic liposomes to practical use has been hindered due to their low stability and potential toxicity *in vivo* [396, 397].

Some of the early work using liposomes of different sizes (40–80 nm, <1 μm, >5 μm, and up to 40 μm) and compositions (Chol/PC, mouse brain homogenates lipids, PC/PS; dioleoyl phosphatidylethanolamine (DOPE) and N-succinyldioleoylphosphatidylethanolamine (SOPE)) failed to show liposomes uptake into the brain in healthy mice or mice with experimental brain metastases [398, 399]. The larger μm-sized liposomes were retained at the BBB possibly fusing with the membranes of endothelial cells and causing embolism and high pressure within the brain, especially in animals with experimental tumors [398, 399]. Interestingly, one of these studies implied that circulating blood monocytes could phagocytose liposomes and deliver them to the brain since these cells were shown to invade experimental metastases even in animals with an intact BBB [398].

It is well known that many liposomes are rapidly cleared from circulation by the reticuloendothelial system, which could decrease the exposure of such liposomes to the BBB after parenteral administration. The immunogenicity of liposomes has also been a concern. Incorporation of PS or phosphatidylinositol in the lipid bilayer in protein-containing

liposomes can stabilize liposomal formulations, prevent the premature protein release in serum and reduce the immunogenicity [400–402]. Decreasing the liposome particle size (< 100 nm) and PEGylation of the liposome surface can also extended the liposomes circulation time. However, such modifications also can reduce liposome interactions with the brain endothelium and neuronal cells thereby hindering delivery of liposomal drugs to the brain. To increase delivery of PEGylated liposomes to the brain, one can modify liposomes with brain-specific ligands that can be internalized and transcytosed across the brain endothelium. For example, modification of the surface of the PEGylated liposomes with transferrin enhanced cellular uptake of the liposomes and delivery of the liposome-encapsulated protein (HRP) in the brain capillary endothelial cells [378]. In an animal study PEGylated liposomes functionalized with brain-targeting ligand, RMP-7 were shown to carry NGF to the brain [379]. Another example of targeted liposomes is so-called "pro-cationic liposomes" modified with a cationic ligand lactoferrin (LF), which targets receptors in brain endothelium [403]. Such modified liposomes were shown to enter the primary rat brain endothelial cells through a combination of adsorptive and receptor-mediated endocytosis. After i.v. administration these liposomes can deliver an encapsulated anticancer agent, doxorubicin to a glioma in a mouse model [404]. In spite of all these findings, the ability of brain targeting moieties to improve liposome delivery to the brain might be questionable. Most of these studies provided evidence using *in vitro* brain endothelial cells. Moreover, most of the animal studies available have not reported brain PK parameters (influx rate, serum half-life etc.) that are essential for evaluation of protein brain delivery outcomes. As a result the interpretation of the effects of some brain targeting moieties can be challenging. For example, one study reported that several brain specific moieties such as angiopep-2, COG133 and CRM197, did not show any improvement in targeting liposomes to the brain *in vitro* and in *in vivo* [380]. Nevertheless, targeting via nutrient transporters expressed at the BBB has been also used to increase brain uptake of liposomes. For example, liposomes modified with reduced glutathione (GSH) have been used as a targeted carrier for delivery of low molecular mass drugs like ribavirin [405], methylprednisolone [406], doxorubicin [407] and an opioid peptide [408]. GSH-liposomes showed higher brain uptake upon i.v. injection in normal rats and in rat model of MS compared to non-targeted liposomes and free drug/peptide, respectively [406, 409]. The i.v. injection of methylprednisolone in GSH-liposomes decreased the clinical score of neurological aberrations in a rat model of MS [406]. GSH-liposomes encapsulating an opioid peptide showed an improved PK profile compared to the free drug in a rat microdialysis study [408]. GSH-PEG modified Doxil® liposomes showed a small but significant increase (compared to non-targeted Doxil® liposomes) in plasma drug concentrations 4 days after i.v. injection in mice; however, drug concentration in the brain was not significantly improved. Nevertheless, these targeted liposomes showed a safety profile similar to Doxil®, decreased brain tumor volume and resulted in a marginal but significant increase in survival time compared to saline and non-targeted Doxil® liposomes in a mouse model of gliablastoma multiforme. Brain delivery of GSH-liposomes was also evidenced using tracer dye carboxyfluorescein as a cargo [410]. The cellular uptake of the dye in rat brain endothelial cells was increased when incorporating to GSH-liposomes compared to that of non-targeted liposomes. Moreover, rat brain microdialysis studies after i.v, injection of GSH-liposomes showed a small but statistically significant increase in the dye concentrations in the brain

extracellular fluid 6 hrs post-injection compared the non-targeted liposomes. However, to the best of our knowledge the GSH-liposomes have not been used for protein delivery to CNS so far.

In addition to a traditional parenteral delivery route liposomes were also administered intranasally. For example, Migliore and colleagues developed cationic liposomal formulation loaded with a model protein, ovalbumin for intranasal delivery [411]. This formulation resulted in elevated brain delivery of encapsulated ovalbumin concentrations in brain compared to delivery of the free protein [411].

Overall, studies on liposomal carriers for protein therapeutics for neurologic diseases have presented conflicting evidence of brain delivery and safety and faced many challenges associated with encapsulation procedures, loading capacity, and preserving protein activity.

## 6.2 PLGA particles

PLGA and its derivatives are used to make perhaps the most widely studied nano/micro-size particles for encapsulation of therapeutic proteins. Advantages of these polymers include their biocompatibility and biodegradability as well as suitability for design of sustained-release protein formulations. The release rates of encapsulated proteins can be tuned by varying the lactic acid to glycolic acid molar ratio and the polymer molecular mass [412]. On the flip side, disadvantages of PLGA carriers include initial "burst" release, irreversible adsorption of proteins to the polymer matrix, as well as inactivation of proteins during preparation, storage and application including effects of products of PLGA degradation, lactic and glycolic acids, on protein stability [413]. Blending with other polymers or excipients, stabilizing proteins during encapsulation by adding zinc or antacid excipients and other means may improve protein stability, loading and release profile [414].

In spite of its extensive use for protein delivery, no direct brain PK data is available showing that PLGA particles improve uptake of encapsulated proteins in the brain. However, a sustained release of proteins from PLGA carriers could benefit the treatment of chronic brain diseases. Indeed, subcutaneous injection of PLGA microspheres containing insulin-like growth factor I (IGF-I) restored the motor function and increased the survival in mice with inherited Purkinje cell degeneration disease [415]. IGF-I was continuously released from the microspheres, which likely increased the brain levels of IGF-I over a period of time and resulted in therapeutic effects similar to a continuous subcutaneous infusion of IGF-I [415, 416]. Another study reported a sustained release for up to 60 days of a therapeutic protein, BDNF from PLGA-poly( L-lysine)-PEG microspheres [417]. Although *in vivo* test was not reported, the bioactivity of the released BDNF was confirmed by cell proliferation and neurite outgrowth in pheochromocytoma PC12 cells stably expressing BDNF cognate receptor TrkB [417].

Interestingly, intracarotid (i.c.) injection of SOD1 encapsulated in PLGA nanoparticles significantly reduced brain infarct volume and prevented neuronal cell death in a rat model of transient ischemic stroke [396]. This study compared 3 different administration routes: i.c., i.v (via the tail and jugular veins) and demonstrated that i.c. route resulted in a 13-fold greater brain uptake of the enzyme compared to the i.v. routes. The observed

neuroprotection could be a result of a sustained release of active SOD1 from nanoparticles, which accumulate in the brain due to the BBB impairment typical of ischemia-reperfusion model.

Like in the case of other carriers, the PLGA nanoparticles can be decorated with brain targeting moieties. For instance, PLGA nanoparticles modified with similiopioid peptide were shown to deliver their payload to the brain after i.v. administration in rats [418, 419]. Notably, the nanoparticles modified with a scrambled peptide did not accumulate in the brain, suggesting involvement of a similiopioid peptide-related brain uptake mechanism [420]. The targeted nanoparticles loaded with a low molecular mass drug, loperamide produced central antinociceptive effect in rats, comparable to the effects of this drug, administered i.c.v.. Another study used PLGA nanoparticles decorated with similiopioid peptide and sialic acid residues, which target sialic acid receptor in brain parenchyma [421]. However, this modification in addition to increased accumulation of the nanoparticles in the brain also appeared to increase their accumulation in peripheral tissues.

Targeting strategies is commonly combined with PEGylation of the nanoparticle surface in an attempt to increase the nanoparticle circulation time and decrease interactions with non-targeted cells. Thus, PEGylated PLGA nanoparticles decorated with tetanus toxin fragment C (a neuron-binding motif) were selectively taken up by neuroblastoma cells but not in hepatocellular carcinoma and BMECs, however, no *in vivo* studies were reported [422]. The PEGylated PLGA nanoparticles conjugated with cationized BSA delivered and released their cargo, 6-coumarin in the brain after caudal vein administration in mice [423]. As is evident from this discussion, most of these studies reported the use of the targeted nanoparticles for the delivery of low molecular mass solutes. However, there are some examples of targeted nanoparticles for the brain delivery of oligo- and polypeptides. For example, PEGylated PLGA nanoparticles decorated with lactoferrin were shown to deliver neuroprotective peptides including S14G-humanin and urocortin to the brain and induce neuroprotective effects in animal models of AD and PD [424, 425]. Overall, although these observations appear encouraging, numerous questions including PK and evidence of brain delivery and release of proteins, as well treatment associated toxicities, in particular immunogenicity of the ligand coated particles, would need to be thoroughly addressed in most cases before a possibility of clinical translation of these systems could be discussed.

### 6.3 PBCA nanoparticles

Kreuter and colleagues evaluated PBCA nanoparticles coated with non-ionic surfactants (polysorbate 80, Pluronic F68) for CNS delivery of a variety of low molecular mass drugs such as doxorubicin, loperamide, tubocurarine, NMDA receptor antagonist MRZ 2/576, and peptides such as dalargin and kytorphin [426]. Later on these nanoparticles were also used to deliver proteins. For example, one study suggested increased brain uptake of NGF and reduced PD symptoms after i.v. administration of NGF-loaded polysorbate 80-coated PBCA nanoparticles in a mouse model of PD [383]. Similarly, Lin and colleagues reported that polysorbate 80-coated PBCA nanoparticles loaded with HRP or enhanced green fluorescent protein (EGFP) can deliver these proteins to the brain in a rat model of TBI [427]. Another study evaluated dextran and polysorbate 80-coated PBCA nanoparticles carrying covalently

immobilized SOD1 and anti-glutamate N-methyl D-aspartate receptor 1 antibody [428]. These protein-PBCA conjugates were shown to prevent neuronal cell death mediated by superoxide radicals $O_2^{\cdot-}$ toxicity in the rat cerebellar cells. No animal study was reported in this work.

The enhanced brain delivery was also observed in PEGylated cyanoacrylate nanoparticles coated with polysorbate 80 [429]. However, not all nanoparticles with polysorbate 80 coating showed enhanced brain delivery. For example, polystyrene nanoparticles coated with polysortabe 80 did not deliver any dalargin cargo into the brain [430]. Instead of brain accumulation, polysorbate 80-coated poly(methylmethacrylate) nanoparticles mainly accumulated in the liver [431]. Olivier and colleagues reported a fast cargo release from PBCA nanoparticles in serum likely caused by polysorbate 80, serum protein competition and rapid nanoparticle degradation in the blood [430, 432].

The brain entry mechanism of PBCA nanoparticles after their i.v. administration is still unclear. It is hypothesized that surfactant-coated PBCA nanoparticles adsorb apolipoprotein E (ApoE) or apolipoprotein B (ApoB) from the bloodstream and cross BBB by LRP-mediated transcytosis [433]. ApoE is a 35 kDa glycoprotein lipoproteins component that plays a major role in the transport of plasma cholesterol in the bloodstream and CNS [434]. Its non-lipid related functions including immune response and inflammation, oxidation and smooth muscle proliferation and migration [435]. Published reports indicate that some nanoparticles such as human albumin nanoparticles with covalently-bound ApoE [436] and liposomes coated with polysorbate 80 and ApoE [437] can take advantage of ApoE-induced transcytosis. Although no studies provided direct evidence that ApoE or ApoB are responsible for brain uptake of the PBCA nanoparticles, the precoating of these nanoparticles with ApoB or ApoE enhanced the central effect of the nanoparticle encapsulated drugs [426, 433]. Moreover, these effects were attenuated in ApoE-deficient mice [426, 433]. Another possible mechanism of transport of surfactant-coated PBCA nanoparticles to the brain is their toxic effect on the BBB resulting in tight junction opening [430]. Therefore, in addition to uncertainty regarding brain transport mechanism of PBCA nanoparticle, cyanocarylate polymers are not FDA-approved excipients and have not been parenterally administered to humans.

## 6.4 Block ionomer complexes (BIC)

BIC (also called "polyion complex micelles") are a promising class of carriers for the delivery of charged molecules developed independently by Kabanov's and Kataoka's groups [438, 439]. They are formed as a result of the polyion complexation of double hydrophilic block copolymers containing ionic and non-ionic blocks with macromolecules of opposite charge including oligonucleotides, plasmid DNA and proteins [438, 440–443] or surfactants of opposite charge [444–449]. Kataoka's group demonstrated that model proteins such as trypsin or lysozyme (that are positively charged under physiological conditions) can form BICs upon reacting with an anionic block copolymer, PEG-poly(α, β-aspartic acid) (PEG-PAA) [440, 443]. Our initial work in this field used negatively charged enzymes, such as SOD1 and catalase, which we incorporated these into a polyion complexes with cationic copolymers such as, PEG-poly( ethyleneimine) (PEG-PEI) or PEG-poly(L-lysine) (PEG-

PLL). Such complex forms core-shell nanoparticles with a polyion complex core of neutralized polyions and proteins and a shell of PEG, and are similar to polyplexes for the delivery of DNA. Advantages of incorporation of proteins in BICs include 1) high loading efficiency (nearly 100% of protein), a distinct advantage compared to cationic liposomes (~32% for SOD1 and ~21% for catalase [450]; 2) simplicity of the BIC preparation procedure by simple physical mixing of the components; 3) preservation of nearly 100% of the enzyme activity, a significant advantage compared to PLGA particles. The proteins incorporated in BIC display extended circulation time, increased uptake in brain endothelial cells and neurons demonstrated in cell culture and increased stability in cells [451]. Our laboratory has demonstrated that BIC-incorporated butyrylcholinesterase (BChE) can be delivered to the brain in BChE–/– mice. Interestingly, the delivery of BChE appeared to be more efficient when the BIC was administered i.m. compared to the i.v. administration [452]. We speculate that BIC administered i.m. could be delivered to the brain via neuromuscular junctions by retrograde transport. In addition, we also developed and characterized several generations of BIC formulations ("nanozymes") of two antioxidant enzymes, SOD1 and catalase and evaluated them in several animal models [451, 453, 454]. For example, a covalently stabilized, cross-linked (*cl*) nanozyme formed by SOD1 and PEG-PLL exhibited improved stability in blood and brain and increased uptake in both brain capillaries and parenchyma, as compared to non-*cl* nanozymes and native protein [453]. The single dose of this nanozyme after i.v. administration resulted in a decreased infarct volume and improved sensorimotor outcomes compared to untreated (saline-injected) and native SOD1 groups in a rat model of transient cerebral ischemia-reperfusion injury. One should expect further developments in evaluation of this new technology for the delivery of proteins to the CNS.

### 6.5 Cell-mediated delivery of nanoparticles

A relatively new approach to CNS protein delivery involves loading of protein-incorporated BIC in immune response cells that respond to pathological inflammation and migrate to the brain tissue thereby serving as conduits for protein delivery [455] (Figure 5). Batrakova and colleagues have investigated this paradigm as a potential strategy for the delivery of therapeutic antioxidant enzymes to treat PD in a series of studies [456–462]. To protect enzymes from degradation in the carrier cells they incorporated these enzymes in the BIC. For example, they loaded catalase-PEI-PEG nanozymes (60–100 nm in diameter) into bone-marrow derived macrophages (BMM) and administered these macrophages i.v. in a mouse model of PD. Nearly 0.5% of protein delivered this way with the BMM accumulated in the brain tissue, which was several fold improvement in brain delivery compared to the nanozymes directly injected in the mouse [462]. The attenuation of PD manifestations (microglial activation and astrocytosis) in animals treated with nanozyme-loaded BMM was also reported, which was not much different from animals injected with the nanozyme alone [462]. The nanozyme-loaded BMM also increased survival of dopaminergic neurons and rescued the loss in the N-acetyl aspartate (used a measure to determine neuroprotection), which suggested the neuroprotective effects. The optimization of the nanozyme formulation for this delivery strategy was also reported [463]. The PK and biodistribution studies demonstrated that nanozyme-loaded BMM had increased area under the curve (AUC), half-life and mean residence time in blood circulation, and greater bioavailability, compared to

nanozyme alone. Enhanced brain delivery of nanozyme loaded in BMM was also demonstrated [464]. However, AUC was also increased (ranging from 1.8 to 4.6-fold) in the non-target organs such as liver, spleen and kidney in addition to the brain tissue. A brain influx rate of 0.026 μL/g.min was determined for nanozyme-loaded BMM, which was 1.9-fold higher than for nanozyme alone. Recent study also suggested that BMM facilitated transport of nanozyme form to brain endothelial, glial and neuronal cells through multiple endocytosis-independent pathways such as transient intercellular connections, macrophage bridging conduits and exosomes [465, 466]. Altogether these studies may open new avenues for cell-mediated protein delivery to the brain. Interested readers are referred to a recent review for a comprehensive overview [467].

## 7. Conclusion

Developing protein therapeutics for treatment of CNS disorders is an unmet need. A variety of delivery strategies discussed in this review have shown promise to delivery proteins to the brain. The most advanced in clinic are the strategies involving direct delivery of proteins to the CNS using the central administration routes, i.c.v. and intraparenchymal, as well as i.t. administration. Recently intranasal administration in the vicinity of nasal cribriform plate, which allows substances to bypass the BBB and enter the brain directly with minimum serum exposure, has gained increasing attention. There appears to be a significant room for advancement of these strategies by combining them with the protein delivery approaches, which were previously explored mainly in the context of the parenteral administration. Modification of proteins with cationic moieties, CPP, fatty acid residues, brain-targeting moieties and amphiphilic block copolymers can result in alteration of protein peripheral PK and increase in the permeability of these proteins at the BBB. Numerous studies demonstrate increased protein uptake in the brain and in some cases increased therapeutic efficacy in relevant CNS disease models. However, the mechanisms of transport of the modified proteins across the BBB in most cases are not well understood. Moreover, there are safety issues associated with most delivery approaches especially the use of cationic moieties and CPP. In contrast, modification of proteins with fatty acids and amphiphilic block copolymers, appear to be promising and in particular, fatty acylation has already reached a clinical stage, although toxicology profiles should be carefully evaluated in each case. Much insight on the mechanism of CNS delivery has been obtained in studies of the PK, pharmacodynamics and toxicity of low affinity antibodies against TfR. In contrast, CNS delivery of proteins using particle-based carriers, in particular, liposomes or PLGA particles appears to be less advanced and less effective. Decorating the particle surface with appropriate molecules that can target receptors at the brain endothelium is one way to address this problem but the available results are still contradictory. Nevertheless, investigating novel nanoparticles to deliver protein to the brain certainly represents a future direction especially in the context of nontraditional delivery approaches avoiding the BBB, intranasal administration and delivery using cells as carriers.

## Acknowledgments

## Abbreviations

| | |
|---|---|
| **Aβ** | amyloid β |
| **Ab-InsR** | antibodies against insulin receptor |
| **Ab-TfR** | antibodies against transferrin receptor |
| **AD** | Alzheimer's disease |
| **Aerosol OT** | bis-(2-ethylhexyl) sulfosucciate |
| **ALS** | amyotrophic lateral sclerosis |
| **ApoB** | apolipoprotein B |
| **ApoE** | apolipoprotein E |
| **ASA** | arylsulfatase A |
| **AUC** | area under the curve |
| **α2-GP** | α2-glycoprotein |
| **Ab-BACE1** | antibodies against β-secretase |
| **BBB** | blood-brain barrier |
| **BDNF** | brain-derived neurotrophic factor |
| **BIC** | block ionomer complexes |
| **BChE** | butyrylcholinesterase |
| **bFGF** | basic fibroblast growth factor |
| **BMECs** | brain microvessel endothelial cells |
| **BMM** | bone-marrow derived macrophages |
| **CED** | convection-enhanced delivery |
| **Chol** | cholesterol |
| **CMC** | critical micelle concentration |
| **CNS** | central nervous system |
| **CPPs** | cell-penetrating peptides |
| **CSF** | cerebrospinal fluid |
| **DIO** | diet-induced obese |
| **DOPE** | dioleoyl phosphatidylethanolamine |
| **DPP-IV** | dipeptidyl peptidase IV |

| | |
|---|---|
| **EPO** | erythropoietin |
| **GDNF** | glial cell-derived neurotrophic factor |
| **GFAP** | gliofibrillar acid protein |
| **GLP-1** | glucagon-like peptide 1 |
| **GSH** | reduced glutathione |
| **EAE** | experimental autoimmune encephalomyelitis |
| **ECS** | extracellular space |
| **EGF** | epidermal growth factor |
| **EGFR** | EGF receptor |
| **ERT** | enzyme replacement therapy |
| **FDA** | Food and Drug Administration |
| **FGF** | fibroblast growth factor |
| **HAD** | HIV-1-associated dementia |
| **HIV-1** | human immunodeficiency virus 1 |
| **HRP** | horseradish peroxidase |
| **HPMA** | N-(2-hydroxypropyl)-methacrylamide |
| **I2S** | iduronate-2-sulfatase |
| **INB** | intranasal-to-brain |
| **i.c.v.** | intracerebroventricular |
| **IGF-I** | insulin-like growth factor I |
| **i.m.** | intramuscular |
| **i.n.** | intranasal |
| **i.t.** | intrathecal |
| **InsR** | insulin receptor |
| **i.v.** | intravenous |
| **KLH** | keyhole limpet hemocyanin |
| **LDL** | low-density lipoprotein |
| **LRP1** | ligand of LDL receptor-related protein 1 |
| **LRP2, LRP1** | ligand of LDL receptor-related protein 2 |
| **LSDs** | lysosomal storage disorders |
| **M6P** | mannose 6-phosphate |
| **MCAO** | middle cerebral artery occlusion model |

| | |
|---|---|
| **MPS I** | mucopolysaccharidosis I |
| **MPS II** | mucopolysaccharidosis II |
| **MS** | multiple sclerosis |
| **NGF** | nerve growth factor |
| **NT-3** | neurotrophin-3 |
| **MTf** | melanotransferrin |
| **NVU** | neurovascular unit |
| **OXM** | oxyntomodulin |
| **PACAP** | pituitary adenylate cyclase activating polypeptide |
| **PBCA** | poly(butylcyanoacrylate) |
| **PBuOx** | 2-butyl-2-oxazoline |
| **PC** | phosphatidylcholine |
| **PCL** | poly(ε-caprolactone) |
| **PEG** | poly(ethylene glycol) |
| **PEI** | poly(ethyleneimine) |
| **PEO** | poly(ethylene oxide) |
| **PET** | position emission tomography |
| **PEtOx** | poly(2-ethyl-2-oxazoline) |
| **PD** | Parkinson's disease |
| **Pgp** | P-glycoprotein |
| **PK** | pharmacokinetics |
| **PLA** | polylactic acid |
| **PLGA** | poly(D,L-lactic-co-glycolide) |
| **PMeOx** | poly(2-methyl-2-oxazoline) |
| **POx** | poly(2-oxazoline)s |
| **PS** | phosphatidylserine |
| **PPG** | polypropylene glycol |
| **PPO** | poly(propylene oxide) |
| **RAP** | receptor associated protein |
| **RDP** | Rabies virus glycoprotein-derived peptide |
| **rhIDU** | α-L-iduronidase |
| **sCAR** | cell surface coxsackie-adenovirus receptor |

| **s.c.** | subcutaneous |
|---|---|
| **Shh** | sonic hedgehog |
| **sMTf** | soluble melanotransferrin |
| **SOD** | superoxide dismutase |
| **SOPE** | N-succinyldioleoylphosphatidyl-ethanolamine |
| **TAT** | trans-activating transcriptional activator |
| **TBI** | traumatic brain injury |
| **TJ** | tight junctions |
| **TfR** | transferrin receptor |
| **TNFR** | tumor necrosis factor decoy receptor |
| **TPGS** | α-tocopheryl polyethylene glycol succinate |
| **VLDL** | very-low-density lipoprotein |

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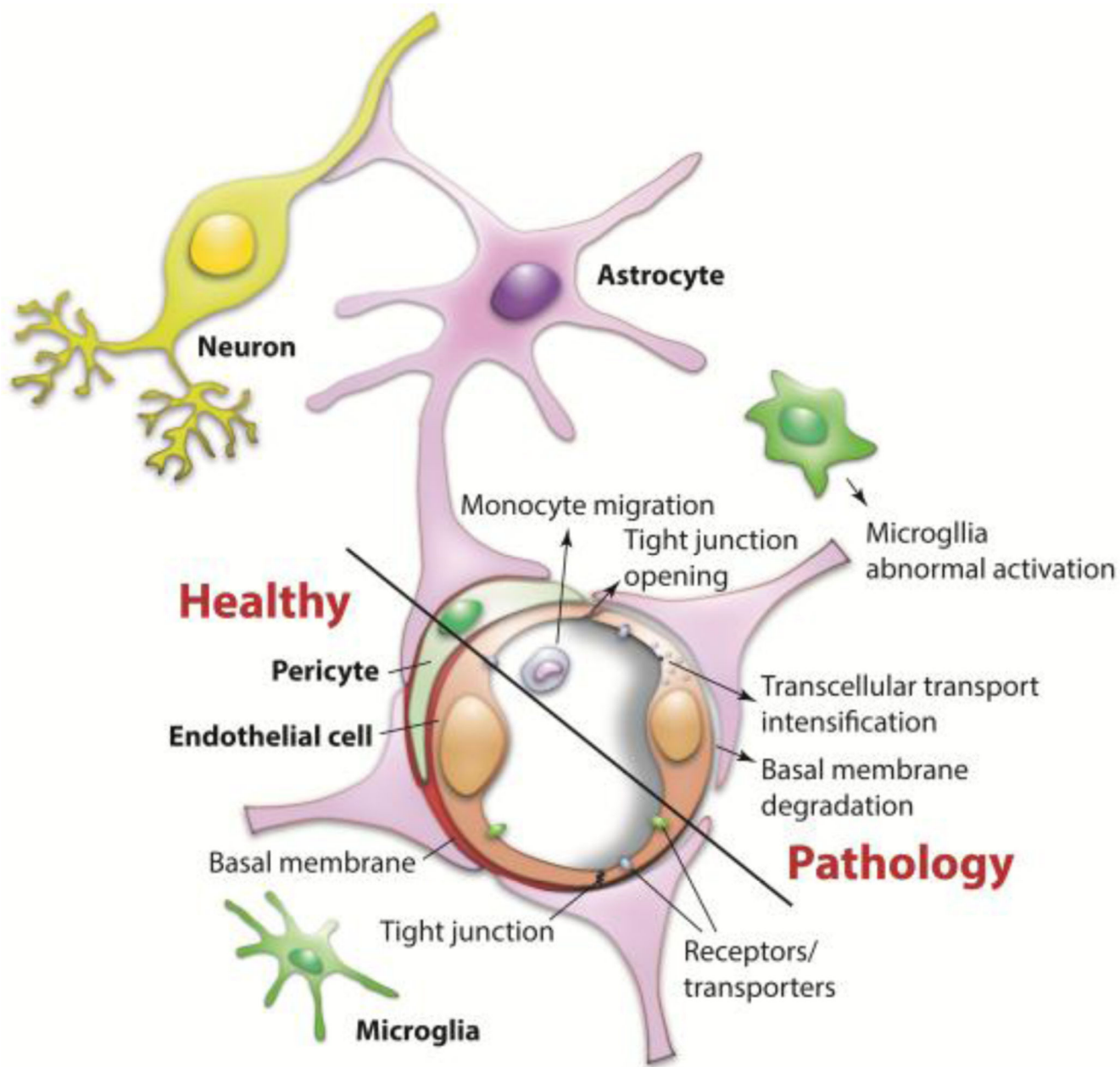
**Figure 1. BBB and NVU in physiological and pathological conditions**
In normal CNS brain microvessel endothelial cells (BMECs) are sealed by tight junction (TJ). They are surrounded at the apical side by a thin basement membrane (BM), which also surrounds the pericytes adjacent to BMEC. Along with astrocytes, microglia and neurons, these cells comprise the neuron vascular unit (NVU). During the disease progression, the TJ can be disrupted and BBB breached [51], BM degraded [30], immune cells migrate from blood to brain, microglia abnormally activated [32], pinocytosis and transcytosis intensified [52, 53], and inwardly and outwardly transporters of various substances modified.
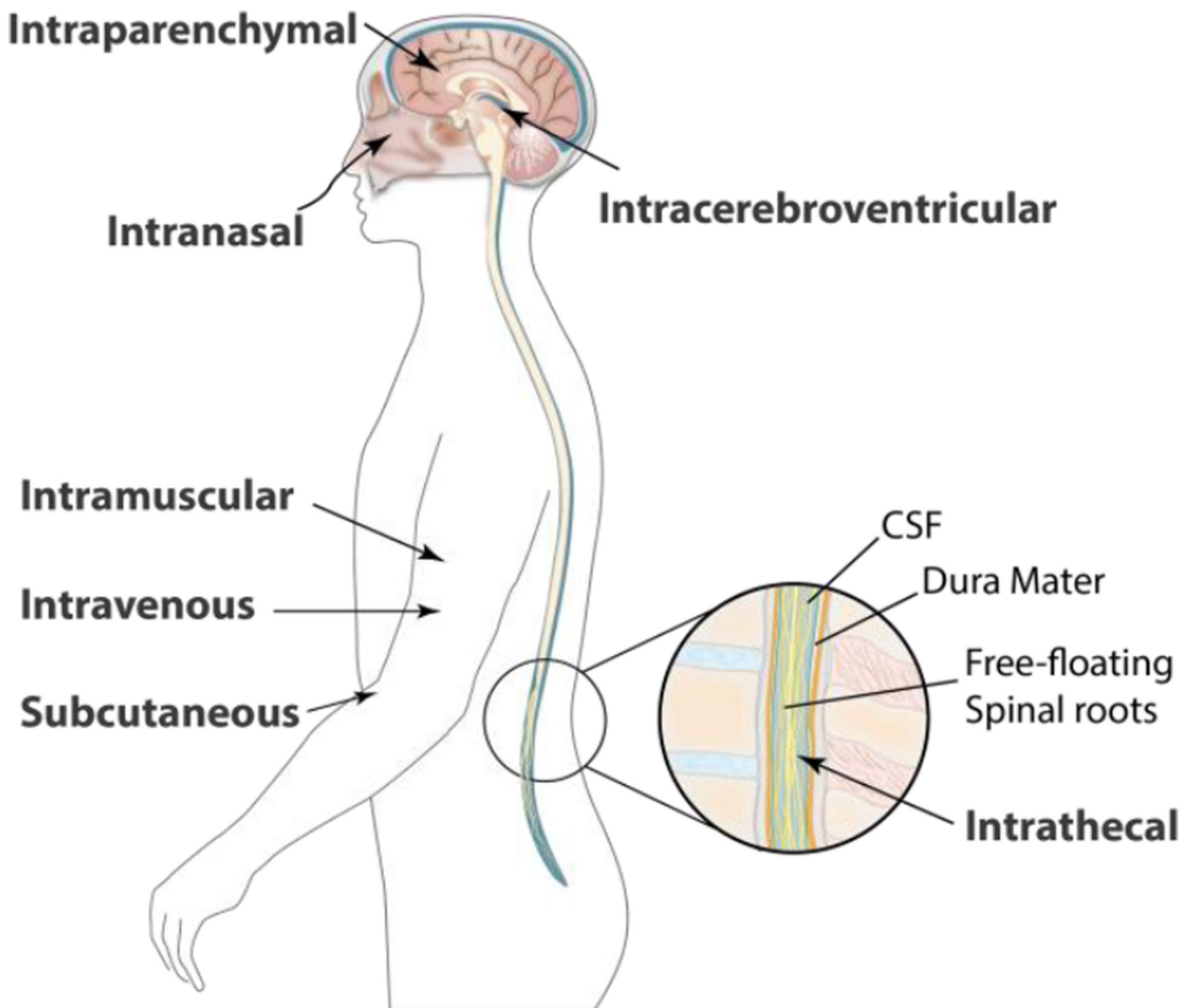
**Figure 2. Administration routes of protein therapeutics for CNS delivery**
Early approaches to CNS protein delivery involved common parenteral injection via intramuscular, intravenous, and subcutaneous routes. In these cases proteins are ultimately distributed within systemic vasculature including brain capillaries. Therefore, brain entry of proteins following parenteral administration requires crossing the BBB. Central administration routes such as intraparenchymal and intracerebroventricular allow bypassing the BBB. Intrathecal administration has been used as a less-invasive alternative to central administration. Intranasal route has been recently explored as a minimally invasive delivery strategy allowing to bypass the BBB. It opens up a new avenue since drug administration at the level of the nasal cribriform plate can facilitate brain delivery with minimal serum exposure.
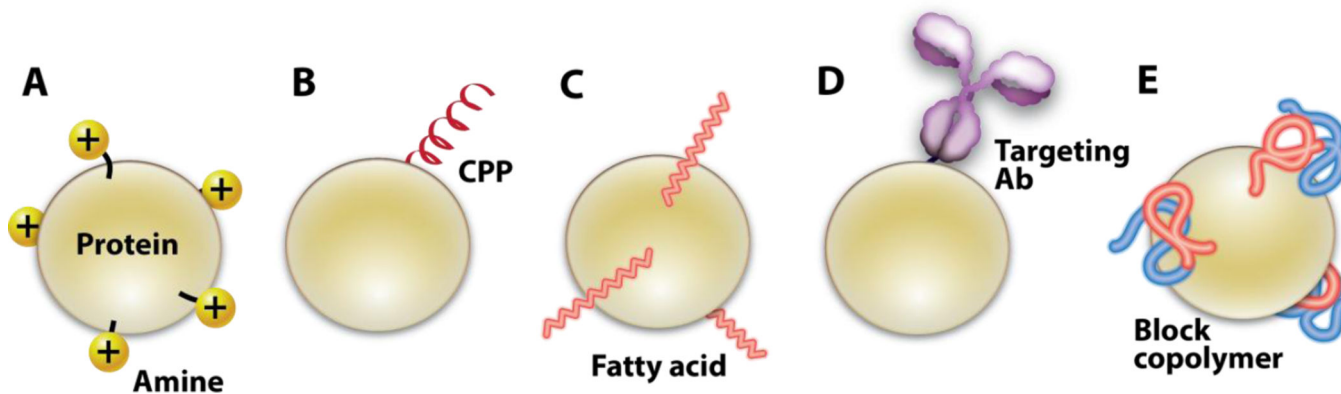
**Figure 3. CNS Delivery of Proteins by Chemical Modification**
**(A) Cationization:** Chemical modification of a protein's carboxylic acid groups with natural or synthetic amines has shown to improve cellular uptake and delivery of the cationized protein into the brain parenchyma. **(B) Fusion protein constructs with CPPs:** CPPs are peptides composed of basic amino acid residues that are believed to traverse across cell membranes thus resulting in intracellular delivery of the attached cargo. **(C) Fatty acid acylation:** This strategy involves modification of proteins with fatty acid residues that increase proteins membrane binding and subsequent cellular uptake. **(D) Conjugation with brain targeting ligands:** This strategy involves conjugation of protein of interest with antibodies or ligands of receptors expressed on the brain endothelium that provide for active transport of the conjugates to the brain. **(E) Modification with amphiphilic polymers.** Pluronic and poly(2-oxazoline) block copolymers have been successfully used to modify proteins to improve their peripheral pharmacokinetic properties and their uptake in the brain parenchyma.
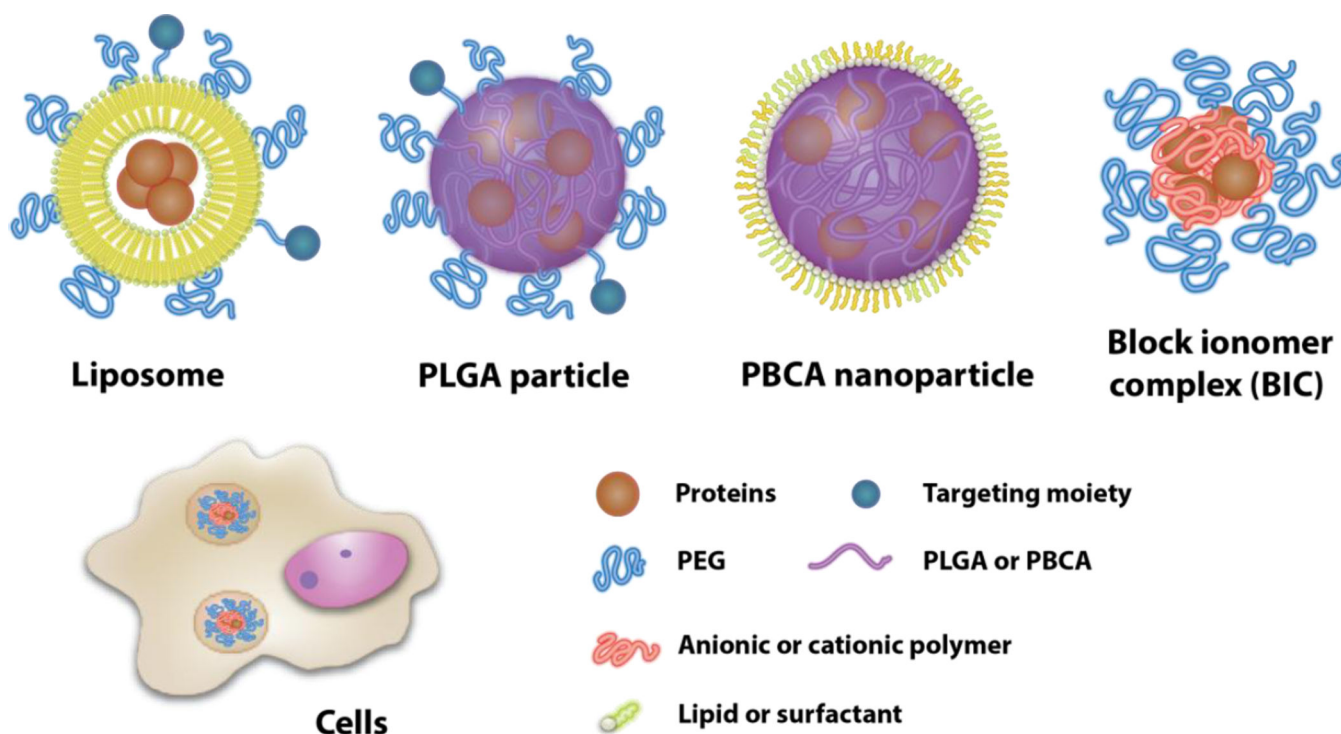
**Figure 4. Particulate Carriers for CNS delivery of Proteins**

**Liposomes** are one early strategy used for delivery of proteins. They have been modified with PEG to increase blood circulation time as well as targeting ligands to increase cellular uptake at the brain endothelium. **PLGA micro- and nanoparticles** have been extensively studied for protein delivery. Their unique advantages include biodegradability and possibility to be used as sustained release formulations. PEGylated PLGA nanoparticles were modified with targeting moieties to improve delivery to the brain. **PBCA nanoparticles** coated with non-ionic surfactants were shown to deliver low molecular mass drugs and proteins to the brain albeit the mechanism of their delivery to the brain is not understood. **BIC** are formed by facile self-assembly of charged proteins and block ionomers of opposite charge. The advantages of this strategy include ease of formulation, high loading efficiency and capacity and retention of protein activity in BIC. Albeit in early stage of investigation this strategy has shown promise in several animal models of disease. **Cell-mediated nanoparticle** delivery is a novel approach that has been successfully demonstrated for delivery of catalase BICs to treat PD in animal models. This strategy involves loading of BICs into the immune cells that respond to pathological inflammation and migrate to the brain tissue thereby facilitating drug delivery.
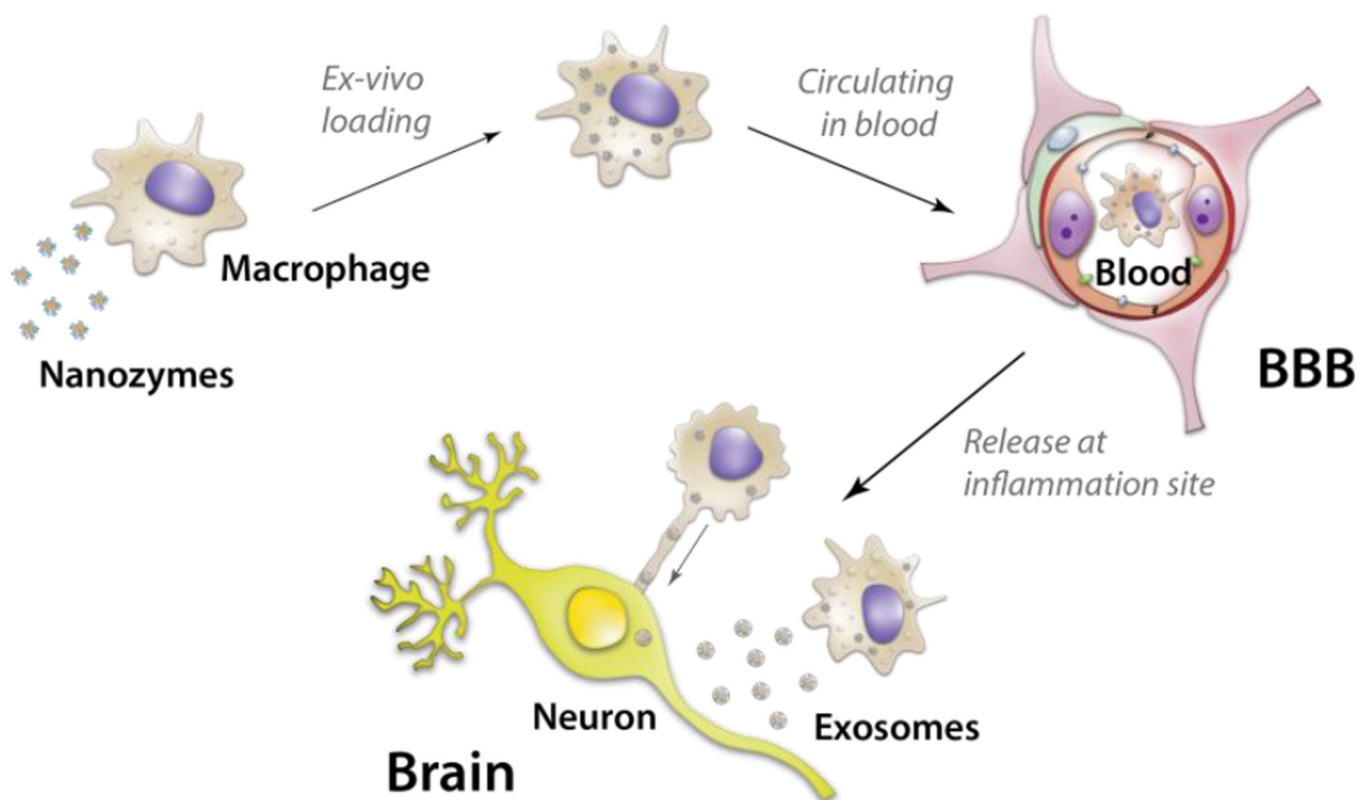
**Figure 5. Cell-mediated nanoparticle delivery to the brain**
Bone marrow-derived macrophages are ex vivo loaded with nanozymes and administered
i.v. into bloodstream. They reach sites of inflammation in the brain, extravasate into the
brain parenchyma and transfer the nanoparticles into brain cells by either 1) directly
transducing surrounding cells (neurons) through cell-cell contacts (e.g. via filopodia) and/or
2) releasing exosomes containing repackaged nanozymes into extracellular space, followed
by the exosomes internalization into brain cells.

**Table 1**

Examples of proteins and peptides with central action that were evaluated in patients

| Compound | Disease indication | Delivery route [a] | Development stage (Trial identifier) | Company or academic sponsor | References |
|---|---|---|---|---|---|
| *Gut-brain hormones* | | | | | |
| Insulin glulisine (APIDRA®) | Cognitive function in AD | i.n. | Phase II (NCT01436045) | HealthPartners Institute for Education and Research | [54–56] |
| Fatty acylated insulin (Humulin® R U-100) | Cognitive function in AD | i.n. | Phase II (NCT01767909) | Alzheimer's Disease Cooperative Study (ADCS) | [54–56] |
| Peptide YY$_{3-36}$ (PYY) | Weight control and safety in obesity | i.n. | Phase II (NCT00537420), failed 2008 | Nastech Pharmaceutical Co. (Merck) | [57] |
| *Lysosomal enzymes* | | | | | |
| β-hexaminidase | Enzyme replacement therapy in GM2 gangliosidoses | i.c.v. | Pilot study, failed 1970s | n/a | [58, 59] |
| Iduronate–2–sulfatase (I2S) | Enzyme replacement therapy in MPS II (hunter syndrome) | i.t. | Phase II (NCT02055118) | Shire | [60, 61] |
| *Neurotrophic factors* | | | | | |
| Nerve growth factor (NGF) | Neurologic function in AD | i.c.v. | Pilot study, failed 1998 | n/a | [62] |
| Glial cell-derived neurotophic factor (GDNF) | Safety, tolerability and motor function in PD | i.c.v | Pilot study, failed 2003 | n/a | [63] |
| GDNF | Motor function in PD | CED to putamen | Phase II, failed 2006 | Amgen | [64, 65] |
| GDNF | Motor function in PD | CED to putamen | Pilot study (UKCRN ID 12085) | North Bristol NHS Trust | |
| Brain-derived neurotrophic factor (BDNF) | Corticoneuronal response in ALS | i.t. | Pilot study, failed 2003 | Montreal Neurological Institute and Hospital, Canada | [66] |
| BDNF | Autonomic sympathetic and parasympathetic function in ALS | i.t. | Pilot study, failed 2005 | Saarland University Hospital, German | [67] |
| *Antibodies* | | | | | |
| Radiolabeled antibody against brain tumor ($^{124}$I-8H9) | Radiotherapy of brain stem glioma | CED to brain stem | Phase I (NCT01502917) | Memorial Sloan-Kettering Cancer Center | [68, 69] |
| *Peptides* | | | | | |
| Synthetic analogue of pituitary hormone 8-arginine vasopressin (DDAVP®) | Antidiuretic replacement management for central cranial diabetes insipidus and temporary polyuria | i.n. | Marketed | Sanofi-Aventis | [70] |
| Oxytocin | Behavior and social function in autism | i.n. | Phase II (NCT01256060, NCT01788072) | Multiple | [71–73] |

| Compound | Disease indication | Delivery route [a] | Development stage (Trial identifier) | Company or academic sponsor | References |
|---|---|---|---|---|---|
| Oxytocin | Satiety Signaling in schizophrenia | i.n. | Phase IV (NCT01614093) | University of Maryland | [74, 75] |

*
i.n. – intranasal; i.t. – intrathecal; i.c.v. – intracerebroventricular; CED – convection enhanced delivery.

**Table 2**

Representative examples of chemical modification to enhance protein therapeutics delivery to the brain

| Strategy | Protein example | Brain PK (proof of concept) | | | | | Efficacy (proof of concept) | Other examples |
|---|---|---|---|---|---|---|---|---|
| | | Method to evaluate brain uptake | Serum T$_{1/2}$ | BBB permeability | Brain uptake | | | |
| Amine | SOD [184, 188] | Permeability coefficient times surface area (PS) and residual brain/ endoneurial plasma volume (Vp) [362] | Dramatic decrease | Drastic increase | n/a | | Following i.v. injection, putrescine modified anti-Aβ$_{1-42}$ ab stained brain vessels in AD transgenic mice; the modified F(ab)2 was detected in parenchyma to bind amyloid deposits [94] | IgG, albumin, insulin [184], NGF, BDNF, NT-3, CNTF [185] |
| TAT | Galactosidase [200] | Immunohistochemistry staining | n/a | n/a | Increase | | Daily i.v. injection of TAT-leptin to mice fed with high fat diet for 19 days showed greater effect on reduction of body weight gain than that of leptin [363] | GDNF [201], Bcl-xL [202], Ngb [364] |
| Fatty acid | HRP [212] | Bidirectional influx rate (Ki) and brain uptake (%inj/g), capillary depletion [365, 366] | No change | Slightly increase | Slightly increase, in parenchyma | | GLP-1 attached by a C16 fatty acid (Liraglutide) displays notable improvement in peripheral PK profile and currently in Phase III as once daily anti-obese drug. No brain PK was reported. [222, 367]. | F(ab)-GFAP, Fab-α2-GP [209], Ghrelin [368, 369] |
| Block copolymer | Leptin [339] | Bidirectional influx rate (Ki) and brain uptake (%inj/g), capillary depletion [365, 366] | Increase | Increase | Increase especially in longer time point | | I.v injection of Pluronic modified leptin is effective in reduction of food intake in obese mice [339] | HRP [212, 335], Anti-GFAP ab, Anti-α2-GP ab [370], SOD [336, 361] |
| Low affinity Ab-TfR | Ab-BACE1 [117, 251, 252] | Brain/serum ratio, brain uptake by radioactivity count and ELISA, autoradiography, imaging | Decrease | Increase | Increase, in parenchyma | | I.v. injected bispecific anti-TfR/BACE1 reduced brain Aβ level in mice [117, 251, 252] | n/a |

| Strategy | Protein example | Brain PK (proof of concept) | | | | Efficacy (proof of concept) | Other examples |
|---|---|---|---|---|---|---|---|
| | | Method to evaluate brain uptake | Serum $T_{1/2}$ | BBB permeability | Brain uptake | | |
| Angiopep-2 | Neurotesin [289] | Volume distribution in brain (Vd) by in situ brain perfusion, imaging | n/a | Increase | Increase, in parenchyma | I.v. injected modified neurotesin resulted in a significant improvement in the analgesic effect for pain control in three rat models of pain [289] | ASA [290], Anti-HER2 ab [287], Anti-EGFR ab [288] |