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Current Strategies for the Delivery of Therapeutic Proteins and Enzymes to Treat Brain Disorders

J.T. Duskey[2], D. Belletti, F. Pederzoli, M.A. Vandelli, F. Forni, B. Ruozi, G. Tosi[1]
[*] Te.Far.T.I., University of Modena and Reggio Emilia, Modena, Italy
[1] Corresponding author: *Email address:* gtosi@unimore.it (G. Tosi)

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Abstract

Brain diseases and injuries are growing to be one of the most deadly and costly medical conditions in the world. Unfortunately, current treatments are incapable of ameliorating the symptoms let alone curing the diseases. Many brain diseases have been linked to a loss of function in a protein or enzyme, increasing research for improving their delivery. This is no easy task due to the delicate nature of proteins and enzymes in biological conditions, as well as the many barriers that exist in the body ranging from those in circulation to the more specific barriers to enter the brain. Several main techniques are being used (physical delivery, protein/enzyme conjugates, and nanoparticle delivery) to overcome these barriers and create new therapeutics. This review will cover recently published data and highlights the benefits and deficits of possible new protein or enzyme therapeutics for brain diseases.

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Abbreviations

DMPC 1,2-dimyristoyl-sn-glycero-3-phosphocholine
DOPE 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
IDUA α-L-iuronidase
α-syn α-synuclein
Ang-2 angiopep peptide-2
ApoB apolipoprotein B
bFGF basic fibroblast growth factor
BBB blood–brain barrier
BMM bone marrow-derived macrophages
CNS central nervous system
CSF cerebral spinal fluid
CTB cholera toxin B
ctCTLA-4 cytotoxic T-lymphocyte antigen 4
EYPC egg-yolk phosphatidylcholine
GDNF glial cell-derived neurotophic factor
GSH-PEG glutathione targeted PEG
GAGs glycosaminoglycans
GFP green fluorescent protein
icam1 intercellular adhesion molecule 1
LINCL late infantile neuronal ceroid lipofuscinosis
MMP9 matrix metalloproteinase-9
MPS mucopolysaccharidosis
MSA multiple system atrophy
MBP myelin basic protein
NEP neprilysin
PPT1 palmitoylprotein thioesterase-1
PBCA polybutylcyanoacrylate
PEG poly ethylene glycol
PLGA poly(lactic-*co*-glycolic acid)
rhSGSH recombinant human sulfamidase
scFV's single-chain fragment variables
TPP1 tripeptidyl peptidase

1. INTRODUCTION

Maladies of the brain lead to some of the most problematic diseases, in terms of symptoms and treatments. Any problem in the brain, disease, or injury related, can lead to communication problems, social

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disorders, organ failure, and death. In 2007, the World Health Organization claimed that ~ 1 billion people are affected with neurological disorders (WHO, 2001). By 2020, 10,000 people annually will be affected by traumatic brain injuries, with another 42 million affected by mild brain injuries, projected to become one of the leading causes of death world wide (Gardner & Yaffe, 2015; Hyder, Wunderlich, Puvanachandra, Gururaj, & Kobusingye, 2007; Ling, Hardy, & Zetterberg, 2015). There is also an increasing rate of well-known diseases such as Alzheimer's (set to increase from 5.4 to between 11 and 16 million cases per year), Parkinson's (50,000 new cases diagnosed each year), autism, epilepsy (50 million per year), and psychiatric illness (suffered by one in four) (MIT, 2014; WHO, 2001). In the United States, an expected ~ 18% of the population will be affected in their lifetime by a mental illness, impartial to age, sex, race, or economic well-being (Fig. 1) (NIMH, 2015).

With such high numbers, social and economic burdens of these diseases are also heavy. In Europe, it was estimated that 35% of the money

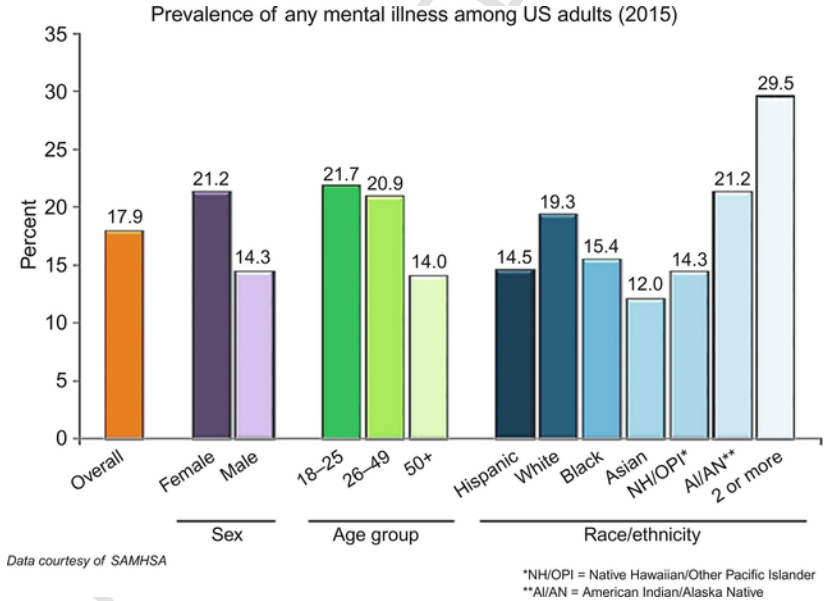

Fig. 1. Occurrence rate of mental illness in the United States (2015). Percent of population divided by common groups including sex, age, and ethnicity for the occurrence of a mental illness. *Modified from NIH website (NIMH. Any mental illness (AMI) among U.S. adults. (2015). U.S. Department of Health and Human Services National Institute of Mental Health. Retrieved from https://www.nimh.nih.gov/health/statistics/prevalence/any-mental-illness-ami-among-us-adults.shtml).*

used for treatments and diseases was due to neurological disorders. This is not only due to rising drug costs and treatments (€798 billion as of 2010) but also due to the loss of time and productivity. The suffering and burden created by these disorders makes it critical to find new methods and improved treatments (MIT, 2014; Muro, 2010).

Discovering, designing, and testing new disease therapeutics is an incredibly difficult task. Millions of years of evolution improved the body's natural ability to defend and purify itself of foreign substances, rendering many therapeutics ineffective (Krol, 2012). Physical barriers-like the skin, filtration system of organs (i.e., liver, kidneys, and spleen), protein and enzyme activities which sequester and degrade foreign objects, and the immune system ensure protection from any foreign substances (Blanco, Shen, & Ferrari, 2015; Finkelstein & Weissmann, 1978; Mitragotri, 2005; Nehoff, Parayath, Domanovitch, Taurin, & Greish, 2014). Therapeutics to treat brain diseases is also subject to extra barriers specifically designed to defend the control center of our bodies. The blood–brain barrier (BBB) controls passage of molecules into and out of the brain through a tightly interconnected cell network (tight junctions), receptor-specific access, and a series of efflux transporters (Chen & Liu, 2012; Gabathuler, 2010; Mikitsh & Chacko, 2014; Misra, Ganesh, Shahiwala, & Shah, 2003; Upadhyay, 2014; Wohlfart, Gelperina, & Kreuter, 2012; Wong, Wu, & Bendayan, 2012). Furthermore, once into the brain parenchyma, the therapeutic must localize into the appropriate region of the brain, enter the correct cell type, and remain active in high enough concentrations for therapeutic effects. Many drug molecules and therapeutics do not naturally pass the BBB or cannot permeate into the brain parenchyma under healthy conditions, but only in some brain diseases which break down these barriers (Bramini et al., 2014; Nau, Sorgel, & Eiffert, 2010; Tosi et al., 2016). Therefore, brain targeted therapeutics should be designed to enter and diffuse without damage (Krol et al., 2012). Very thorough and extensive literature describing the BBB, the inability for many therapeutics to permeate the subcompartments of the brain, and the numerous other barriers of brain delivery exist and will not be discussed here (Alyautdin, Khalin, Nafeeza, Haron, & Kuznetsov, 2014; Bhaskar et al., 2010; Garg, Bhandari, Rath, & Goyal, 2015; Lu et al., 2014; Mitragotri, Burke, & Langer, 2014; Patel, Zhou, Piepmeier, & Saltzman, 2012; Singh & Kapil, 2011; Wohlfart et al., 2012).

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Some small molecules have shown some therapeutic effectiveness but many brain diseases are caused by a protein or enzyme deficiency (Yi, Manickam, Brynskikh, & Kabanov, 2014). Therefore, research of protein and enzyme therapeutics to correct the deficiency has increased. While replacing the defective protein/enzyme is a logical and natural option, numerous variables can lead to unsuccessful treatment making it a challenging task (Peluffo et al., 2015). Proteins and enzymes are more susceptible to systemic and brain barriers (protein binding, first pass clearance, immune response, attraversing the BBB, and off-target effects). Not only must they be delivered in high quantities, they must also remain intact, and for enzymes, the 3D orientation, and folding of the enzyme must be conserved (Calias, Banks, Begley, Scarpa, & Dickson, 2014; Miners, Barua, Kehoe, Gill, & Love, 2011; Pardridge, 2015; Yi & Kabanov, 2013).

There are currently several main categories in which delivering proteins or enzymes to the brain are being persued. These include physical methods, systemic delivery, protein or enzyme conjugates, and nanoparticle-based delivery. This review will focus on describing the most recent avenues to treat brain diseases with therapeutic proteins or enzymes, and to highlight the benefits as well as the shortcomings of each.

2. PHYSICAL DELIVERY

Therapeutics and treatments for many diseases have rapidly progressed over the last century, while common methods for treating brain diseases have unfortunately remained somewhat barbaric. Instead of elegantly overcoming the numerous barriers such as the BBB, efflux pumps, tight junctions, and highly selective receptors present to block therapeutic delivery to the brain, many current treatments still rely on a direct injection to circumvent these obstacles (Gabathuler, 2010). Unfortunately, many drugs do not diffuse freely through the brain cavity requiring separate injections to reach all affected sites, making the difficulty, aggressiveness, and possibility for permanent damage of direct injection a serious concern (Glascock et al., 2011; Nau et al., 2010; Tosi et al., 2016; Wolak & Thorne, 2013); However, this method, while crude, holds many advantages in the treatment of brain and central nervous system (CNS) disorders. Direct injection can specifically target the brain compartment and cells of interest removing the loss of first pass clearance and off-tar-

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get toxicity. Moreover, the stability and pharmacokinetic half-life of the protein or enzyme are less critical because they arrive directly at the site of action (Gabathuler, 2010; Marshall et al., 2015).

Vast numbers of articles are still being published to characterize direct injections into the different compartments of the CNS, and the effects and limitations of dosing various types of therapeutics in the brain (Marshall et al., 2015; Vuillemenot et al., 2011; Vuillemenot et al., 2015). Recently, Beard et al. tried to deconvolute the different effects of the recombinant human sulfamidase enzyme (rhSGSH) in the brain after administration into cerebral spinal fluid (CSF) compartments: intrathecal lumbar, cisternal, and ventricular (Beard et al., 2015). Understanding brain distribution is critical for improving currently tested therapeutics against diseases such as various lysosomal disorders, diabetes, blocking drug efflux (Cannon, Peart, Hawkins, Campos, & Miller, 2012), and correcting damage to the CNS (a more comprehensive list can be found in Table 1).

While these treatments are still some of the most effective and highly sought after options, they are subject to many deficits. A major limiting factor is the invasiveness of inserting an object into the brain. Tearing a hole in the BBB increases chance of infection in the brain as well as increasing the chance of causing irreparable damage (Meng et al., 2014). This is often compounded by the need for improved circulation of the injected therapeutic throughout the brain compartments for effective treatment (Beard et al., 2015; Dickson et al., 2007; Marshall et al., 2015). This requires higher volumes/doses, and/or multiple injections, possibly into multiple locations. One option to extend effects and achieve higher volume doses in the brain is administration by infusion. The benefits of perfusion include controlled dosing speed and time, localization within the brain by bypassing the blood and filtration organs, ability to control the contents of the solute (including potential proteins or enzymes that could interfere), and the ability to analyze efflux rates by changing solutions (Cannon et al., 2012; Fujikawa et al., 2010; Katz et al., 2014; Smith & Allen, 2003). In general, both direct injections and infusions are not viewed favorably by the public and are used due to a lack of other options. This becomes even more important when the age of onset of many brain disorders is taken into account. Many diseases, such as Alzheimer's, Parkinson's, and brain cancer are more predominant in older patients. Other diseases, such as late infantile neuronal ceroid lipo-

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TABLE 1 Physical Delivery to the Brain

Year	Therapeutic	Malady[a]	Therapeutic	Methods Tested[b]	Model	Conclusion	Citation
2007	Enzyme	Hurler syndrome	Recombinant human iduronidase (rhIDU)	IT	Canine	IT rhIDU diffused widely throughout the CNS. Functional with a clinically applicable injection frequency and dose	Dickson et al. (2007)

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TABLE 1 (Continued)

Year	Therapeutic	Malady[a]	Therapeutic	Methods Tested[b]	Model	Conclusion	Citation
2010	Protein	Diabetes	Leptin	ICV infusion	Mice	Icv leptin delivery was not specific to any brain structures. The identities of the neurons mediating leptin's anti-T1D effects are still unknown	Fujikav Chuang Sakata Ramad and Coppa (2010)

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TABLE 1 (Continued)

Year	Therapeutic	Malady[a]	Therapeutic	Methods Tested[b]	Model	Conclusion	Citation
2011	Enzyme	Late infantile neuronal ceroid lipofuscinosis (LINCL)	Tripeptidyl peptidase-1 (TPP1)	Four IT administrations formulated in artificial cerebrospinalfluid (aCSF)	Canine	Spike in TPP1 concentration in the CSF and circulation. However there was a higher immune response in afflicted mice. Improvements in brain morphology and cognitive function were not detected	Vuillemenot et al. (2011)

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TABLE 1 (Continued)

Year	Therapeutic	Malady[a]	Therapeutic	Methods Tested[b]	Model	Conclusion	Citation
2012	Enzyme	MPS II (Hunters syndrome)	Iduronate-2-sulfatase (I2S)	ICV and ITL	Mice, canine, and primates	Both ICV and ITL led to extensive distribution of the enzyme. The data suggest enzyme distribution could depend on unknown active transfer processes	Calias et al. (2012)

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TABLE 1 (Continued)

Year	Therapeutic	Malady[a]	Therapeutic	Methods Tested[b]	Model	Conclusion	Citation
2014	Enzyme	Lysosomal storage diseases	Recombinant human sulfamidase (rhSGSH)	IV, intraspinal CSF infusion	Canine	Penetration after IT injection was suboptimal earlier and longer treatments must be tested	Marshall et al. (2015)

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TABLE 1 (Continued)

Year	Therapeutic	Malady[a]	Therapeutic	Methods Tested[b]	Model	Conclusion	Citation
2014	Enzyme	Neuronal ceroid lipofuscinosis (CLN2)	Tripeptidyl peptidase-1 (TPP1)	ICV catheter, ITL catheter	Canine	Detectable delay of neurological deficits and disease progression was slowed. Canines exhibited improved performance on a cognitive function test, reduced brain atrophy, and increased life span	Katz et al. (2014)

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TABLE 1 (Continued)

Year	Therapeutic	Malady[a]	Therapeutic	Methods Tested[b]	Model	Conclusion	Citation
2014	Enzyme fusion protein	MPS IIIB	IGFII modified α-*N*-acetylglucosaminidase (NAGLU)	ICV	Mice	Administration led to an almost complete reduction of heparin sulfate and a complete reversal of pathology within 2 weeks. However large amounts of enzyme were found in the liver even after ICV dosing	Kan et al. (2014)

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TABLE 1 (Continued)

Year	Therapeutic	Malady[a]	Therapeutic	Methods Tested[b]	Model	Conclusion	Citation
2015	Enzyme	Neuronal ceroid lipofuscinosis (CLN2)	Recombinant human tripeptidyl peptidase -1 (rh-TPP1)	ICV catheter	Canine	rhTPP1 was detected in all areas of the CNS leading to decreased lysosomal storage accumulation, and improved CNS cellular phenotypes. While an increased immune response was observed, authors claimed an improved safety profile	Vuillemenot et al. (2015)

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TABLE 1 (Continued)

Year	Therapeutic	Malady[a]	Therapeutic	Methods Tested[b]	Model	Conclusion	Citation
2015	Enzyme	MPS IIIA	Recombinant human sulfamidase (rhSGSH)	IT, cisternal, and ventricular	Mice	Widespread, corrective amounts of rhSGSH after 1 week occurred only when dosed in the lateral ventricle. However, because this strategy is more invasive than intrathecal lumbar injection validation is required in larger animal models	Vuillemenot et al. (2011)
2015	Enzyme	Neuronal ceroid lipofuscinoses (NCLs)	Palmitoylprotein thioesterase-1 (ppt1)	IT	Mice	Increased delivery led to improved rotarod results and increased survival with no observable toxicity	Lu et al. (2015)

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2015	Enzyme	Spinal cord damage	Sonic hedgehog (Shh)	Injection into the contusion site	Rats	Dosing did not negatively impact the progression of a spinal cord injury but only led to a minor decrease in scaring compared to free enzyme. Further optimization is required		Rauck, Novos Oudeg and W (2015)

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2016	Enzyme	MPS III	Recombinant human sulfamidase (rhSGSH)	Osmotic pump-assisted delivery to the right lateral ventricle	Mice	The enzyme was stable under infusion pump conditions. Over 13 weeks a return to normal levels of heparin sulfate was observed in both hemispheres of the brain and the cervical spinal cord	King et al. (2016)

[a]Mucopolysaccharidosis (MPS).
[b]IT, Intrathecal; ICV, intracerebroventricular; ITL, intrathecal lumbar; IV, intravenous.

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fuscinosis (LINCL), Hurler syndrome, Krabbe disease, and many other lysosomal storage diseases are treated in young children, as the diseases often become fatal at a young age (Beard et al., 2015; Calias et al., 2012; Lu et al., 2015; Vuillemenot et al., 2011). The risk of permanent damage during treatment is greatly increased at young or older ages. Finally, the major drawback of these treatments currently is they often do not lead to a cure for the disease. Authors often express that while concentration in the desired region is enhanced, amelioration of the symptoms is not achieved. Furthermore, even if a therapeutic effect is observed, it only retards the progression of the symptoms as opposed to a permanent fix. This has led scientists to search for new, less invasive methods which can be used in addition to current treatments to provide safer and longer lasting therapeutic options.

Other physical delivery methods have also gained interest. Ultrasound can be used to create a therapeutic response. Ultrasound waves create oscillations in different tissues and lead to increased temperatures eliciting a biological response (Dasgupta et al., 2016; Hernot & Klibanov, 2008; Mitragotri, 2005; Vykhodtseva, McDannold, & Hynynen, 2008). More so, these waves can lead to cavitation of bubbles leading to a sheering force, which upon bursting the bubbles can create microjets that can stress or make tears in the tissue (Dasgupta et al., 2016; Park, Zhang, Vykhodtseva, & McDannold, 2012). Ultrasound has now been used in numerous applications for the delivery of various types of therapeutic agents, mainly in organs other than the brain. In 2012, Wang et al. successfully delivered glial cell-derived neurotrophic factor (GDNF) conjugated-biotinylated lipid-coated microbubbles to the brain of rats (Wang et al., 2012). In this study, the protein delivery to the brain was increased ~ 10-fold compared to GDNF microbubbles without sonication. However, the authors also noticed a very large amount of BBB disruption. While, to the best of our knowledge, sonoporation has not been used for enzyme delivery to the brain, it has previously been used to increase enzyme activity targeted to the heart, seen in 2005 with the delivery of active luciferase into rat cardiac tissue (Bekeredjian, Chen, Grayburn, & Shohet, 2005). This highlights its possible use for neurodegenerative diseases and brain delivery. This strategy is highly desirable due to the ability to directly modify microbubbles with different ligands and therapeutics, which when combined with more sensitive and tunable sonoporation techniques, leads to a very elegant and highly targeted technique (Ma et

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al., 2016; Xu et al., 2016). However, its inherent physical nature is a concern. The strong force upon destroying the microbubbles, leads to high amounts of disruption of the BBB hindering its protective function (Vykhodtseva et al., 2008). This force could also have negative effects on the activity or structure of the therapeutic protein and enzyme cargo leading to less efficient treatment options.

3. NONPHYSICAL METHODS

Physical methods have become more strategic and better understood; however, they still suffer from serious drawbacks for treating brain diseases. The danger, destructive nature, lack of brain distribution, and lack of symptom correction of physical methods make researching more elegant, functional, and mild methods of delivery critical (Beard et al., 2015; Marshall et al., 2015; Rauck et al., 2015). Nonphysical methods are highly sought after such as viral- or nanoparticle-based delivery. Not only do these suffer from all the barriers as physical methods such as the cell membrane, distribution in the brain, and short therapeutic duration, they must also be designed to overcome first pass clearance, instability in the blood, immune response, and off-target effects (Azad et al., 2015; Chen & Liu, 2012; Lu et al., 2014; Upadhyay, 2014; Yi et al., 2014). To this end, the protein or enzyme must be protected to inhibit degradation, avoid protein binding in the blood (i.e., stealthed with poly(ethylene glycol) (PEG)), avoid clearance by the liver, have an extended pharmacokinetic half-life, and be targeted (peptide, antibody, aptamer, etc.) to cause brain accumulation and minimize off-target effects (Duskey & Rice, 2014). While often in conflicting, all of these parameters must be optimized for any chance of therapeutic effect in clinical trials.

3.1. Systemic Delivery

Injection of proteins and enzymes into the blood stream is hindered by many factors. Both are naturally bound by blood proteins and removed by first pass clearance. Even if the molecule circulates in the blood, it is often degraded by proteases or is lost to off-target accumulation (Duskey & Rice, 2014). However, the severity of these diseases warrants any possible treatments to be examined. To overcome these barriers with systemic administration, one possibility is to perform multiple doses or dras-

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tically increase the dose of the therapeutic, effectively saturating systemic clearance but leading to enhanced accumulation in all tissues. One example was treating the lysosomal storage disease mucopolysaccharidosis type I (MPS1), an autosomal recessive brain disorder leading to death often before 10 years of age (Ou, Herzog, Koniar, Gunther, & Whitley, 2014). A major barrier to treat this disease is that α-L-iduronidase (IDUA) does not readily cross the BBB. Therefore, researchers drastically increased the amount dosed intravenously in hopes that the small percent of such a large dose that can cross the BBB would be enough to see therapeutic effects. At a dose of 11.6 mg/kg/week in mice, levels of the enzyme increased to almost wild-type levels, reducing ganglioside levels by 63% and improving their water T-maze test results. While beneficial, the drastic increase in treatment costs to purify large quantities of enzyme and increased risk to the patients raise serious concerns about the feasibility of this kind of treatment in clinical trials. Increased accumulation in the other tissues, in this instance the heart, taxing the blood filtration organs, an increased possibility for an immune response, and adverse health effects cannot be tolerated. To this end, in 2015 a known treatment for children over six with Hunter's syndrome (Idursulfase beta: Hunterase®) was administered consecutively over 52 weeks to determine the safety profile of repeat dosing (Sohn et al., 2015). Treatment led to a significant decrease in glycosaminoglycans (GAGs) of all patients, and none showed adverse reactions to the enzyme itself. However, ~ 16% of patients showed adverse reaction to the repeated perfusion process and two-thirds of patients showed antibodies against Hunterase®. Direct injection, large concentrations, and repeat doses are all short-term treatment options, but it is critical to find more stable and less aggressive alternatives. This requires a more sophisticated approach involving modified, conjugated, or encapsulated proteins and enzymes delivering therapeutic levels of enzymes to be specifically delivered to the brain while avoiding the barriers of clearance, binding, degradation, and off-target toxicity.

3.2. In Vitro Targeting

Scientists are incessantly searching for new ways to successfully treat brain diseases with less physically invasive methods. Therapeutic molecules are designed to optimize their stability, pharmacokinetic profile, and biodistribution in numerous animal models. Without each of these

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points being successful, it is impossible to create a viable therapeutic treatment. However, overcoming all of these barriers at once is an arduous task, and often can be separated by using relevant in vitro models. In vitro cell experiments are very useful, with various animal and human cell models for a wide array of diseases, either naturally occurring or created through gene knockout technology, including: Parkinson's, Alzeihmer's, lysosomal storage disorders, a wide variety of brain cancers, etc. Along with a large array of fluorophores, reporter molecules, and assay techniques available (Elisa, Western, protein pull down, fluorescent microscopy, etc.) in vitro experiments can give valuable data in a therapeutic molecules potential. Much more simplistic than an animal model, cell models are an effective way to more quickly analyze therapeutic factors. With an intact cell and nuclear membrane which express the proteins and receptors like normal cells, it is possible to quickly develop and screen compounds for their ability to enter the cell, either through nonspecific uptake or receptor-mediated entry, and test their activity. In recent years, in vitro assays have been invaluable for discovering targeting ligands or cell penetrating peptides which increase therapeutic entry across the BBB (Georgieva, Hoekstra, & Zuhorn, 2014; Malakotikhah, Teixido, & Giralt, 2008; Malakoutikhah, Guixer, Arranz-Gibert, Teixido, & Giralt, 2014; Oller-Salvia, Sanchez-Navarro, Giralt, & Teixido, 2016; Pardridge, 2015; Steichen, Caldorera-Moore, & Peppas, 2013; Teixido & Giralt, 2012). More specifically, large screenings of different ligands (i.e., small molecules, peptides, proteins, etc.) have been analyzed for the ability to deliver enzyme cargo into various cell types (Acosta, Ayala, Dolan, & Cramer, 2015; Eiamphungporn, Yainoy, & Prachayasittikul, 2014; Gramlich et al., 2016). One such example used fluorescent-labeled cholera toxin B (CTB) to detect GM1-gangliosidosis and intercellular adhesion molecule 1 (icam1) to deliver enzymes into fibroblasts (Rappaport, Garnacho, & Muro, 2014). While in vitro assays are useful for screening for activity and stability, alone they are severely flawed in designing enzyme and protein therapeutics that function in animal models or humans. Even with the cell barriers, in vitro assays lack the stresses found during circulation and crossing the BBB. Promising new models have emerged to more accurately mimic delivery to the BBB in vitro (Janigro, Leaman, & Stanness, 1999; Wilhelm & Krizbai, 2014). However, strong in vitro results rarely translate equally in vivo. This problem was addressed recently by Zuchero et al. who stated that litera-

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ture cited targets discovered in microarray screening rarely led to increased uptake in the brain (Zuchero et al., 2016). Using proteomic analysis, they identified protein targets on brain endothelial cells. Furthermore, they tested antibodies against these targets in mice to ensure increased brain accumulation. While useful at optimizing new protein and enzyme therapeutics, in vitro data alone are severely lacking and require simultaneously validation in vivo.

3.3. In Vivo Proteins, Fusion Proteins, and Modified Enzymes

The possibility for protein and enzyme therapeutics to overcome circulation, and uptake while remaining active is very narrow. Proteins and enzymes can be modified with stabilizing or targeting moieties to overcome these barriers. In recent years, peptide-modified enzymes have been extensively tested for enhancing brain delivery. For example, a peptide derived from the protein transduction domain of the human immunodeficiency virus protein (TAT), angiopep peptide (Ang-2), and variants of the apolipoprotein (ApoB, and ApoE-I and II) were used to deliver arylsulfatase A to a diseased mouse model (Bockenhoff et al., 2014). While all of these peptides enhance cellular uptake, ApoE-II-modified enzyme showed the largest accumulation in the brain, limiting clearance from the liver and spleen when compared with unmodified. However, the decrease in sulfidase storage was still far below that of wild-type mice. More interesting, Meng et al. showed these peptides also worked to deliver the enzyme tripeptidyl peptidase 1 (TPP1) when free in solution with the enzyme (not covalently bound) (Meng et al., 2014). The ability for these types of peptides to induce cell uptake has been known for a long time. In 1999, groups were already attempting to deliver β-glucosidase to the brain with peptides such as TAT (Schwarze, Ho, Vocero-Akbani, & Dowdy, 1999). While the number of peptides to enhance uptake has dramatically increased, the fact remains that very few have led to viable therapeutics over 2 decades and necessitates the design of more complex options.

Another option is to use a protein to activate the immune system against brain pathologies. Searching for a treatment for Alzheimer's and other dementia, one group dosed antibodies against the phosphorylated tau in diseased mouse models (Boutajangout, Ingadottir, Davies, & Sigurdsson, 2011). Results showed that phf1 antibody arrived in the dentate gyrus and motor cortex. This translated to a decrease in tau levels as

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well as improved traverse beam score results; however, no observable difference was seen in the other functional tests. In another mode, the cytoplasmic domain of cytotoxic T-lymphocyte antigen 4 (ctCTLA-4) was conjugated to a novel cell penetrating peptide for delivery to T-cells (Lim et al., 2015). This antigen downregulated the T-cells which are the cause of inflammation in multiple sclerosis, and when inhibited led to a significant improvement in uptake as well as clinical scores in a mouse model.

Fusion proteins are naturally occurring proteins, incorporating portions of various proteins into one molecule. Recent reviews summarize the concept, possibilities, and techniques for creating synthetic fusion proteins (Watts & Dennis, 2013; Yu, Liu, Kim, & Lee, 2015). Researchers can synthetically replicate this concept with the possibility to modify the physical, as well as functional characteristics of proteins, creating a potential candidate to target and treat brain diseases (Ou-Yang et al., 2015; Wang et al., 2012). One such result was to use a lentiviral vector to create a fusion protein of the LDL-R region of ApoB with single-chain fragment variables (scFVs) against α-synuclein (α-syn) (Spencer et al., 2014a). Upon dosing the purified protein, not only did the fusion protein show a 20–30-fold increase of brain accumulation compared with the native antibody, uptake into neurons, and improved water maze results in mice, but the ability to produce the protein in mammalian cells greatly reduced the risk of immunogenic responses compared to those made in bacterial models.

Other advances combining fusion proteins with enzymes consisted of the Igg fusion protein containing an antibody targeted to the human insulin receptor (HIRMab) to deliver N-sulfoglucosamine sulfamidase (SGSH) for the treatment of mucopolysaccharidosis type IIIA in monkeys (Boado, Lu, Hui, & Pardridge, 2014). In vitro models showed an 83% decrease in sulfate levels, and when injected in vivo results showed an enhanced accumulation in the brain with therapeutic relevance (1%). While 1% of dose was considered enough for corrective effects it is critical to optimize new formulations to increase brain accumulation. This will not only lead to better treatments but will also decrease doses and limit costs for future therapeutics.

Not only are fusion proteins a more sophisticated therapeutic molecule, but the mode of delivery also impacts their potential to treat brain disease. One method to enhance the fusion proteins therapeutic potential

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relied on plant cells to produce and bioencapsulate fusion proteins (Mäger, Roberts, Wood, & El Andaloussi, 2014). Kohli et al. produced a fusion protein containing myelin basic protein (MBP) via a furin-cleavable linker to the CTB subunit in plant cells (Kohli et al., 2014). This production created the fusion protein within chloroplasts, protecting them during oral administration. Upon release the CTB portion promoted transcytosis across the intestinal epithelium and allowed rapid blood and organ delivery where MBP acted as the therapeutic reducing amyloid fibril formation found in Alzheimers' patients. When visualized with green fluorescent protein (GFP) only a small portion entered the brain, but the delivery of MBP led to a 70% decrease in amyloid formation in mouse brain and retina. This method provides an intriguing solution to overcoming the barriers of brain delivery and is also unique due to its ability to be dosed orally.

Another interesting delivery method is genetically engineered modified enzymes in viral vectors. Although viral vectors usually fall into the category of nanoparticles and in general are not being discussed in this review, a few warrant mentioning here. Instead of using the viral vector to directly deliver therapeutics to the brain, labs engineered them to produce modified enzymes in vitro which were injected systemically. One such example against Alzheimer's disease used purified enzyme from cells infected with a virus coding the protease neprilysin (NEP) modified with the brain targeting moiety ApoB (Spencer et al., 2014b). Compared with the control vehicles, the targeted enzyme showed more than 10-fold increased localization with the neurons, as well as correction of symptoms to almost normal levels. Equally important, the mice showed very little immunogenic response to the dosed enzymes.

Removing the need and costs of protein purification, researchers took this idea one step further. By dosing the viral vectors directly to transfect a "depot organ" (usually the liver) for enzyme production and release into the blood circulation (Spencer et al., 2015). Two recent articles used this method to treat disease models of mucopolysaccharidoses type IIIA or multiple system atrophy (MSA) (Sorrentino et al., 2013; Spencer et al., 2015). In both cases, the accumulation in the brain was greatly enhanced and symptoms were almost completely corrected. This method had some of the most promising data seen to date for the ability to correct symptoms of a brain disorder and for its potential translatability to human clinical trials; however, great care must be taken as once the or-

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gan is transfected, the quantity of enzyme produced, as well as the immune response goes unmediated with potentially dangerous side effects.

Further research to reduce off-target uptake, enhance brain specificity, incorporate other therapeutics, and test them in higher animal models is necessary to find potential cures. All of these delivery methods have great promise in correcting these issues in mouse models and will dramatically impact future therapeutics against brain diseases.

3.4. Nanoparticle-Based Delivery

Nanoparticles offer a unique method to simultaneously combine multiple moieties into the same therapeutic for successfully treating brain diseases. The chemical and biological flexibility of nanoparticles is almost second to none compared with the other delivery methods. There are many types of nanoparticles based on lipids, polymers, dendrimers, inorganic nanoparticles, etc. (Young, Stenzelb, & Yang, 2016) (Fig. 2). Reviews of general delivery with various nanoparticle types are abundant in the literature (Finkelstein & Weissmann, 1978; Kreuter, 2014; Mahapatro & Singh, 2011; Mignani et al., 2016; Posadas, Monteagudo,

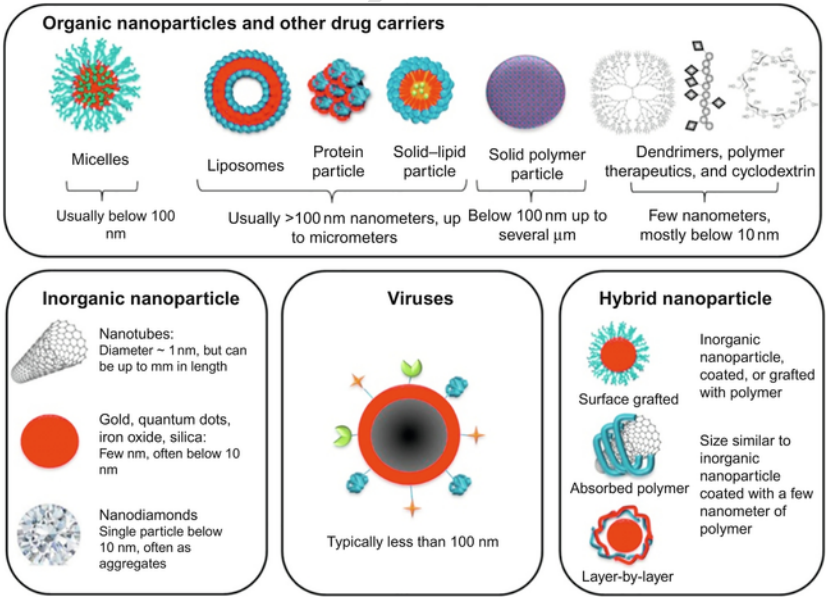

Fig. 2. Categories of nanoparticles. *Reproduced from Young, S. W. S., Stenzelb, M., & Yang, J.-L. (2016). Nanoparticle-siRNA: A potential cancer therapy? Critical Reviews in Oncology/Hematology, 98, 159–169 with Copyright permission.*

& Cena, 2016; Tosi, Costantino, Ruozi, Forni, & Vandelli, 2008). Depending on the core material, it is possible to finetune almost all physical characteristics of nanoparticles including: size, shape, charge, binding capacity, and hydrophobicity/hydrophilicity (internal and external). These factors correlate to the nanoparticle pharmacokinetic half life, biodistribution, stability, and binding capacity for the therapeutic protein or enzyme. Furthermore, many of these nanoparticles are suitable for further modification with targeting ligands or stealthing agents such as PEG. With so many possibilities, researchers can combine variables to optimize the particle for each individual protein, enzyme, and brain disease.

Recently protein only nanovectors have been tested for brain delivery (Estrada, Chu, & Champion, 2014). In the literature protein, conjugates of this type accumulated in higher amounts than monomers alone (Serna et al., 2016). Serna et al. modified GFP with the peptide brain targeting ligands, ang-2 or seq, and a cationic tail (Serna et al., 2016). The ang-2 proteins remained as monomeric units under all conditions; however, the cationic tail aided in the formation of nanoparticles for the seq containing proteins. The theory and formation of these particles are significant because these could be translated directly to most if not all therapeutic proteins. However, upon evaluation in healthy mouse models, the targeted nanoparticle accumulation in the brain was indistinguishable to non-targeted. The difference between previous literature successes and lack of accumulation in the brain exemplifies the increased difficulty in delivering to the BBB over other organs. In a different approach, carbon nanospheres were covalently linked to the nuclear matrix-binding protein SMAR1 to treat a mouse model of human CNS demyelinating diseases, including MS and acute disseminated encephalomyelitis (Chemmannur, Bhagat, Mirlekar, Paknikar, & Chattopadhyay, 2016). In this study, the modified carbon nanospheres showed no toxicity across all concentrations tested as well as uptake into proinflammatory th17 cells. This induced a decrease in interleukins as well as an improved clinical score compared with the nanospheres alone over 19 days, but unfortunately long-term experiments were not conducted. While this method did not directly target the disease in the CNS, the nanospheres induced an effect in one part of the body that led to a therapeutic response in the brain in an autoimmune disease. This technology would be very interesting to

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combine with other targeted therapeutics to have additive/synergistic effects.

Poly(lactic-*co*-glycolic acid) (PLGA) is a biodegradable and biocompatible polymer approved by the US Food and Drug Administration for human use. This polymer forms nanoparticles which have been used to delivery various proteins to the brain (Fornaguera et al., 2015). In a series of articles, PLGA nanoparticles were directed to the brain either by coating them with polysorbate or targeted with a peptide ligand (g7) (Chaturvedi, Molino, Sreedhar, Khrestchatisky, & Kaczmarek, 2014; Salvalaio et al., 2016). In both cases crossing the BBB was enhanced in comparison to nontargeted molecules, but only delivery was tested and further investigations into therapeutic relevance are required.

Liposomes were also used to delivery proteins to ameliorate the symptoms of Alzheimer's (Rotman et al., 2015). Glutathione-targeted PEGylated (GSH-PEG) liposomes formulated from either 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) or egg-yolk phosphatidylcholine (EYPC) were loaded with amyloid beta binding llama single-domain antibody fragments (VHH-pa2H). In this way, the GSH-PEG helped particles cross the BBB, and upon destruction, release the antibody fragments targeting the amyloid plaques. Both types of liposomes, loaded with antibody fragments, greatly increased the pharmacokinetic half-life and brain uptake in an Alzheimer's mouse model compared to the antibody fragments alone; approximately 10-fold higher for GSH-PEG-EYPC (the better of the two formulations).

These results exemplify the positives and negatives of nanoparticle delivery to the brain. While any increase in therapeutic accumulation is a victory, and a 10-fold increase is substantial, when the percentage of dose was measured even the best formulation only led to ~ 0.023% of the injected dose in the brain. With such a low percent arriving in the brain and a lack of data testing the therapeutic effect, its relevance is minimal. Therefore, it is important to continue to improve targeting nanoparticles to deliver larger, therapeutically relevant amounts of protein into the brain.

Another route to enhance protein-loaded nanoparticle delivery to the brain is nasal delivery of drugs into the brain (Lin et al., 2016; Lochhead & Thorne, 2012). This method is less invasive than direct injection, but has many specific requirements for use as a viable delivery method (Appu, Arun, Krishnan, Moffett, & Namboodiri, 2016). Like direct injec-

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tion, nasal delivery has the advantage of skipping first past clearance and can lead to distribution of the therapeutic to various CNS regions via access through the olfactory bulb and brainstem. This is exemplified by delivery of chloramphenicol acetyltransferase to various parts of the brain (Appu et al., 2016). When coupled with matrix metalloproteinase-9 (MMP-9), a molecule known to permeabilize the nasal epithelium, delivery was increased twofold to the midbrain and cortex, and ~ 3-fold to the brainstem. However, nasal delivery also has its faults. As reported, "the major disadvantage of nasal drug delivery is the limited absorption across the nasal epithelium and inadequate transport to the affected brain tissues. Although this can be overcome with permeation enhancers… the potential toxicity to the mucosal surface after repeated use of permeation enhancers has restricted their application" (Zhao et al., 2016). To this end, the same authors demonstrated the possibility for lipid nanoparticles loaded with basic fibroblast growth factor (bFGF) to treat a stroke model in rats (Zhao et al., 2016). Results showed that compared to free bFGF, accumulation in the brain was improved in the olfactory bulb, pallium, hippocampus, and striatum. This increased delivery also led to drastic decrease (~ 50%) in stroke infarct size and an extended life expectancy up to 21 days. Another group compared exosome delivery by intranasal vs intravenous injection (Haney et al., 2015). By fluorescent imaging, it was clear that intranasal delivery created a much higher rate of uptake than with intravenous delivery. Further analysis showed a decreased amount of activated microglial cells to levels near control mice. More information on the treatment of Parkinson's disease with exosomes was also covered recently in a review by the same group (Wu, Zheng, & Zhang, 2016). These advances have shown the possibility to improve this method for functional use; however, are still limited in clinical use requiring new improved for advancing treatment of brain diseases (Garcia-Corvillo, 2016).

The physical characteristics of nanoparticles also make them suitable to encapsulate larger cargo like enzymes. There are many cases in which enzyme-loaded nanoparticles are tested for stability, cell uptake, and activity; however, few have been designed specifically for brain delivery (Lee et al., 2014; Ortac et al., 2014). One group compared different types of nanoparticles: liposomes, polybutylcyanoacrylate (PBCA), or PLGA, targeted with anti-NMDA (*N*-methyl-D-aspartate) receptor 1 antibody (anti-NR1-SODPBCA NPs) (Yun et al., 2013). Although the amount of

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antibody varied greatly between vehicle type, all particles showed brain accumulation leading to a 50% reduction in infarct size in an ischemic mouse model. In another example of liposomes, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were modified with GNeo–NHS. In this way, both positive and negatively charged cargo could be encapsulated (Hamill, Wexselblatt, Tong, Esko, & Tor, 2016). When loaded with a-L-iduronidase and delivered to fibroblast cells, a significant decrease in glycosaminoglycans was observed. Good uptake, loaded with functional enzyme, and the ability to encapsulate both positive and negative cargo permits the direct translation of these particles to other enzymes and diseases; however, effective targeting to better cross the BBB will be necessary to advance in vivo.

Another interesting targeted brain delivery method involved treating bone marrow-derived macrophages (BMM) with nanoformulated (PEI-PEG) catalase ex vivo (Zhao et al., 2011). The BMMs were then injected via tail vein or jugular vein and a dramatic increase in pharmacokinetic half life of the enzyme was observed. Furthermore, the BMMs naturally accumulated at sites of inflammation. This led to an increased accumulation in the brain over 7 days in a brain inflammation model. This was followed up by another paper in which they modified BMMs with GDNF leading to a significant decrease in inflammation and neurodegeneration in a Parkinson mouse model (Zhao et al., 2014). This method has very promising features because not only does it target the site of inflammation, and protect the enzyme, but also it could be personalized using BMMs from the patient themselves limiting the possibility for an immune response.

Nanoparticles offer the largest number of controllable variables of all therapeutic options ranging both in physical characteristics, and possible therapeutic options. Because of this, it is often very difficult to draw conclusions about the relevance of the data in comparison to the others. Each system offers different positive features, and currently none have stood out as having a clear advantage for clinical success. Future works will continue to require combining little victories from each piece of research to optimize and to find viable protein and enzyme-based treatments for brain diseases.

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4. CONCLUSION

Brain disease rates are increasing worldwide at an astounding rate. They will soon become one of the most deadly and expensive medical costs in the world. Unfortunately, there are currently very few successful treatments. Because many of these diseases can be traced to a missing or deficient protein or enzyme, finding new ways to protect and specifically deliver these molecules into the brain need to be discovered. To be effective, these new therapeutics must bypass the initial biological barriers including: administration, sequestration and degradation in the blood, first pass clearance, off-target accumulation, and the immune response. Adding to the difficulty, they must also cross the BBB, diffuse through the brain compartments and be able to enter the deficient cells to create a therapeutic effect.

Research has uncovered many avenues in which we can approach the treatment of brain diseases using therapeutic protein or enzyme replacement therapy. These range from physical methods, conjugates, or nanoparticle delivery. Each offers unique advantages over the other. Direct injection can deliver intact proteins and enzymes into the brain by bypassing almost all major physical barriers; however, it is extremely invasive, compounded often by the need for repeated treatments. Proteins and enzyme conjugates can be used to increase stability, targeting, crossing the BBB, and cell penetration, but often fail to combine them all into one functional system. The versatility of nanoparticle-based delivery improves on direct conjugation by affording protection and further functionalization options to increase the pharmacokinetic half life, targeting, and delivery, but still falls short of creating a complete system in which all variables are combined. These disadvantages have drastically limited the number of clinical trials and successful therapeutics that have reached the market.

For future research, combining treatments and methods are necessary. For example, combining physical methods with nanoparticles, advancing bioconjugate technologies to enhance the delivery, stability, pharmacokinetics, delivery across the BBB, and diffusion through the brain compartments while simultaneously stabilizing the protein or enzyme are necessary. Only if all of these factors are combined into a single delivery vehi-

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cle will it be possible to create a clinically relevant protein or enzyme therapeutic for brain diseases.

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