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Brain delivery of biotherapeutics *via* receptor-mediated transcytosis across the blood–brain barrier

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The brain microvasculature represents the blood–brain barrier (BBB) *in vivo* and it is permeable only to small lipophilic molecules. Polar nutrients, like glucose and amino acids, are transported across the BBB *via* carrier-mediated transport systems. Large protein-based biotherapeutics are not able to cross the BBB, which has represented a major issue for the development of potential treatments for the central nervous system over the past several decades. The finding that proteins such as insulin and transferrin cross the BBB through receptor-mediated transcytosis (RMT) led to the idea that it may be possible to transport peptidomimetic molecules to the brain by targeting these BBB receptors. It was later demonstrated that monoclonal antibodies (MAb) targeting either insulin and transferrin BBB receptors were able to penetrate the BBB and distribute throughout the brain. A first generation of molecular Trojan horses or shuttle systems were developed, which were able to piggyback therapeutic molecules conjugated directly to these MAbs or bound to them *via* avidin–biotin chemistry. This technology was also applied to the delivery of genes and antisense oligonucleotides to the brain. A second generation of brain penetrating protein-based biotherapeutics was produced in a form of fusion proteins, comprised of a transport domain and a therapeutic domain. These fusion proteins were validated in various experimental models, including lysosomal storage disorders, stroke, Parkinson's and Alzheimer's disease, respectively. Clinical trials with brain penetrating fusion proteins have been completed or are in progress with valanafusp alpha and lepunafusp alfa for Hurler's syndrome (mucopolysaccharidosis type I, MPS I), with pabinafusp alfa and tvidenofusp alpha for Hunter's syndrome (MPS II), and with trontinemab for Alzheimer's disease. Pabinafusp alfa was the first brain penetrating biotherapeutic approved by a regulatory agency for the treatment of Hunter MPSII syndrome. The aim of this article is to review the progress made in the brain delivery of biotherapeutics *via* RMT across the BBB.

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1. Introduction

The brain microvasculature represents the blood–brain barrier (BBB) *in vivo*, and its biological characteristics have been extensively reviewed.^{1–7} The BBB is primarily permeable to lipophilic molecules of less than 400 Da.^{1–3,7} Thus, hydrophobic small molecules, like histamine, do not cross the BBB.^{2,7} Polar nutrients of low molecular weight, like glucose and amino acids, are transported across the BBB *via* carrier-mediated transport (CMT) or facilitated transporters. For example, the glucose transporter type 1 (GLUT1) is the BBB transporter for glucose, while large neutral amino acid transporter (LAT1) mediates the transport of large neutral amino acids.^{8,9} Proteins in general, do not cross the BBB. However, there are exceptions, like insulin, transferrin, leptin, and insulin-like

growth factor.^{10–13} These proteins are produced in peripheral organs, and they are transported across the BBB *via* specific receptors that induce receptor-mediated transcytosis (RMT) through the brain microvasculature.^{10–13} The hypothesis that peptidomimetic drugs can be transported into the brain *via* these BBB RMT systems was elaborated in the mid 1980s.^{10,14} A series of species-specific monoclonal antibodies (MAb) directed against either human insulin receptor or rodent transferrin receptor were produced, which are reviewed in section 2.^{15–17} These MAbs were able to bind their respective BBB receptor without interfering with its natural ligand and induced RMT across the BBB^{15–19} (Fig. 1). In the early 1990s, the pharmacological efficacy of brain-penetrating conjugates of a nerve growth factor and the vasopressin intestinal peptide (VIP) was demonstrated *in vivo*.^{20,21} A brain penetrating form of VIP was produced by conjugation to the OX26 anti-rat transferrin MAb, and this fusion construct markedly increased the brain blood flow.²¹ On the contrary, the VIP alone had no effect in the brain, as it does not cross the BBB.²¹ Other chemi-

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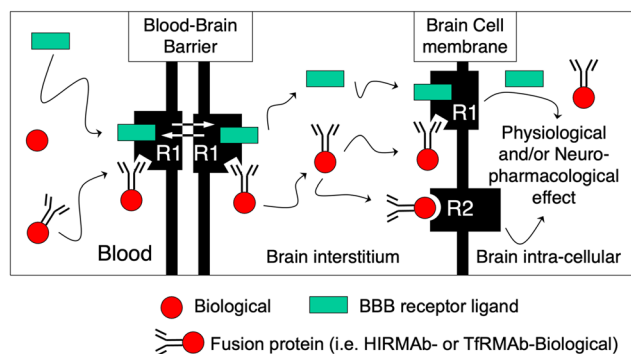


Fig. 1 Transport of brain penetrating IgG across the BBB via RMT. IgGs and/or IgG fragments target an exofacial epitope of endogenous BBB receptors (R1), i.e.: insulin and/or transferrin, without interfering with the binding of the natural ligand (green rectangle) and/or the physiological transport across the BBB by RMT. Potential biotherapeutics for the CNS, like enzymes, MAb, decoy receptors, and/or neurotrophic factors (red circle) do not cross the BBB and stay in circulation following IV administration. These therapeutic agents can be re-engineered as a fusion protein, or a chemical conjugate, with a brain penetrating IgG or IgG fragment to piggyback on these transport systems. Depending on the therapeutic domain of the IgG-fusion protein, the construct may: (1) bind to its ligand in the brain interstitial compartment (as in the case of bispecific MAbs or decoy receptors); (2) target a brain cell membrane receptor (R2), as for neurotrophic factors; or (3) be endocytosed via the same targeted R1 receptor in brain cells, as is the case for lysosomal enzymes or antisense oligonucleotides, thus producing a physiological and/or neuropharmacological effect. Abbreviation: HIRMAb, monoclonal antibody (MAb) directed to the human insulin receptor; TfRMAb, MAb directed to the transferrin receptor. Reproduced from ref. 37 with permission from MDPI, copyright 2022.

cal conjugates of MAb targeting the transferrin and insulin receptors were reported and its efficacy demonstrated in rodents and non-human primates.^{22–25} These conjugates

include brain penetrating forms of antisense oligonucleotides, siRNA and non-viral gene delivery systems, and they are reviewed in sections 3.1 and 3.2. The cloning of brain penetrating MAbs allowed for the genetic engineering of molecular Trojan horses or shuttle systems, and the production of a generation of fusion proteins comprised of a transport domain and a therapeutic domain.^{26–31} The former targets a BBB receptor that induce RMT across the BBB, and the latter exert the pharmacological effect in brain. Numerous fusion proteins have been engineered and its efficacy demonstrated *in vivo* in experimental models of lysosomal storage disorders, stroke, Parkinson's and Alzheimer's disease, respectively (section 3.3, Tables 1 and 2). Clinical trials have been completed or are in progress with the brain penetrating fusion proteins valanafusp alpha and lepunafusp alfa for Hurler's MPSI syndrome, pabinafusp alfa and tvidenofusp alfa for Hunter's MPS II syndrome and trontinemab for Alzheimer's disease.^{32–35,53} Pabinafusp alfa was the first brain penetrating biotherapeutic approved by a regulatory agency for the treatment of Hunter's MPSII syndrome.³⁶ The Academia and the Biotech Industry continue to be focused on the development of biotherapeutics for brain exploiting the RMT systems of the BBB. The aim of this article is to review the progress made in the brain delivery of biotherapeutics *via* RMT across the BBB.

2. Design brain penetrating MAbs

2.1. Production of brain penetrating MAbs

A first generation of MAbs directed against BBB receptors that induced RMT included the murine 83-14 and 83-7 MAbs targeting the human insulin receptor (HIR), the mouse OX26 MAb directed against the rat transferrin receptor (rTfR), and the rat 8D3 and R17 MAbs targeting the mouse TfR (mTfR).^{15–17} The TfRMABs were species specific, and the

Table 1 Brain penetrating IgG-fusion proteins targeting the BBB human insulin receptor

Indication	Therapeutic domain	IgG-fusion protein ^a	Ref.
Hurler syndrome (MPS I)	Iduronidase (IDUA)	HIRMAb-IDUA (valanafusp alpha)	32
Hunter syndrome (MPS II)	Iduronate-2-sulfatase (IDS)	HIRMAb-IDS	38
Hunter syndrome (MPS II)	IDS	HIR-Fab-IDS	39
Metachromatic leukodystrophy ^b	Arylsulfatase A (ASA)	HIRMAb-ASA	40
Sanfilippo A (MPSIIIA) ^b	Sulfamidase (SGSH)	HIRMAb-SGSH	41
Sanfilippo B (MPSIIIB) ^b	N-Acetyl-alpha-D-glucosaminidase (NAGLU)	HIRMAb-NAGLU	42
Niemann-Pick A/B ^b	Acid shingomyelinase (ASM)	HIRMAb-ASM	43
Tay-Sachs ^b	Hexoaminidase A (HEXA)	HIRMAb-HEXA	43
Batten type 1 ^b	Palmitoyl-protein thioesterase (PPT1)	HIRMAb-PPT1	43
GM1-gangliosidosis ^b	β-Galactosidase (GLB1)	HIRMAb-GLB1	43
Alzheimer's ^b	Anti-Aβ amyloid single chain Fv antibody (scFv)	HIRMAb-Aβ bi-specific antibody	44
Parkinson's, ALS, Alzheimer's, and/or stroke ^b	Tumor Necrosis Factor Decoy Receptor (TNFR)	HIRMAb-TNFR	45
Parkinson's, Alzheimer's, and/or Friedreich ataxia ^b	Erythropoietin (EPO)	HIRMAb-EPO	46
Parkinson's, stroke, and/or drug/EtOH addiction ^b	Glial cell derived neurotrophic factor (GDNFf)	HIRMAb-GDNF	47
Stroke, neural repair ^b	Brain-derived neurotrophic factor (BDNF)	HIRMAb-BDNF	48
Organophosphate exposure	Paroxonase-1 (PON1)	HIRMAb-PON1	49
Various	Any mono-biotinylated therapeutic	HIRMAb-avidin	50

^a The transport domain of these human fusion proteins is a monoclonal antibody (HIRMAb), or a Fab fraction (HIR-Fab-IDS), directed to the human BBB insulin receptor. The indication and the therapeutic domain of the fusion protein are listed for the corresponding IgG-fusion protein. ^b Represents an indication that has a primary CNS disease burden.



Table 2 Brain penetrating IgG-fusion proteins targeting the mouse or human BBB transferrin receptor

Indication	Therapeutic domain	IgG-fusion protein ^a	Ref.
Hurler syndrome (MPS I)	Iduronidase (IDUA)	mTfRMAB-IDUA	51
Hunter syndrome (MPS I)	IDUA	hTfR-Fab-IDUA (lepunafusp alfa)	33
Hunter syndrome (MPS II)	Iduronate-2-sulfatase (IDS)	hTfRMAB-IDS (pabinafusp alfa)	34
Hunter syndrome (MPS II)	IDS	mTfRMAB-IDS	52
Hunter syndrome (MPS II)	IDS	TfR transport vehicle, ETV : IDS (DNL310) (tvidenofusp alfa)	53
Sanfilippo A (MPSIIIA) ^b	Sulfamidase (SGSH)	mTfRMAB-SGSH	54
Alzheimer's ^b	Anti-A β amyloid single chain Fv antibody (scFv)	mTfRMAB-A β bi-specific antibody	55
Alzheimer's ^b	Anti-A β amyloid MAb	TfR-scFab-A β -MAb (trontinemab)	35
Alzheimer's ^b	Anti-A β amyloid MAb	TfR-tetavalent bispecific RmAb158-scFv8D3	56
Parkinson's, Alzheimer's, and/or stroke ^b	Tumor Necrosis Factor Decoy Receptor (TNFR)	mTfRMAB-TNFR	57
Parkinson's, Alzheimer's, and/or stroke ^b	Erythropoietin (EPO)	mTfRMAB-EPO	58
Parkinson's, and/or stroke ^b	Glial cell derived neurotrophic factor (GDNF)	mTfRMAB-GDNF	59
Various	Any mono-biotinylated therapeutic	mTfRMAB-avidin	60

^a The transport domain of these fusion proteins is a monoclonal antibody directed to the mouse BBB-transferrin receptor (mTfRMAB) or the human BBB transferrin receptor (hTfRMAB). The indication for humans or experimental mouse models and the therapeutic domain of the fusion protein are listed for the corresponding IgG-fusion protein. ^b Represents an indication that has a primary CNS disease burden.

HIRMAB cross-reacted with Old World primates, such as the Rhesus monkey.^{15–19} Similar to their endogenous ligands (*i.e.*: insulin and transferrin), these MABs target the appropriate BBB receptor with high affinity, *i.e.*: dissociation constants (K_D) in the low nM range, but without interfering with the normal transport of the ligands across the BBB.^{15–19} This generation of MABs enabled experimental testing of the hypothesis that potential biotherapeutics could be transported across the BBB by piggybacking on these RMTsystems.^{20–25} The brain uptake of various conjugates and fusion proteins was 1–3% of the injected dose (ID)/brain,³⁷ which is comparable to the brain uptake observed of lipid soluble small molecules such as diazepam.⁶¹ The brain volume of distribution (VD) of the brain penetrating biotherapeutics ranged from ~300 to 800 $\mu\text{L g}^{-1}$.³⁷ As a reference, the brain VD of a non-BBB penetrating IgG is equivalent to the blood volume in brain, which is 14–20 $\mu\text{L g}^{-1}$.^{38,62} The extent of BBB penetration of these MABs was quantified using the capillary depletion technique. This method showed that the VD in the brain parenchyma was comparable to the one in the post-vascular supernatant. These findings demonstrated that the majority of the fusion protein penetrated the BBB and was not retained in the brain microvasculature.^{37,38,62} HIRMAB and TfRMAB conjugated to peptides and antisense oligonucleotides *via* the avidin-biotin technology demonstrated brain penetration and therapeutic efficacy (section 3.1.). HIRMAB and TfRMAB were also used to deliver genes to the brain with immunoliposomes (section 3.2.). Subsequently, a new generation of brain penetrating biotherapeutics targeting the BBB HIR and TfR was developed by genetic engineering (Tables 1 and 2) (section 3.3.).

Other MABs directed to the TfR were produced, and new hypotheses were postulated involving the affinity and valency of the MAB to produce shuttle systems with improved brain penetration. It was postulated that low-affinity monovalent MAB directed to the BBB TfR transport system may result in improved brain uptake as compared to the conventional high affinity TfRMAB.⁶³ This hypothesis is based on the observation

that bivalent high affinity TfRMABs may cause clustering of the TfR and triage of the antibody-TfR complex to the lysosome and degradation of TfR and/or recycling back to the luminal plasma membrane of the endothelial cell.⁶³ However, this observation was based on experiments performed with TfRMAB-avidin fusion proteins in tissue culture experiments, which are known to form tetrameric structures from the association of avidin monomers.^{64,65} No *in vivo* toxic effects have been reported for other high-affinity TfRMAB fusion proteins. The chronic treatment with intravenous (IV) TfRMAB-GDNF (2 mg per kg body weight [BW]) twice weekly for 12 weeks did not downregulate BBB TfR, as the pharmacokinetics of this fusion protein was virtually identical before and after the chronic treatment.⁶⁶ No evidence of BBB TfR downregulation was reported either in a chronic study performed with pabinafusp alfa, the high-affinity human bivalent TfRMAB-IDS fusion protein, in the cynomolgus monkey with doses up to 30 mg per kg per week for 26 weeks.⁶⁷ In addition, numerous bivalent high affinity TfRMAB fusion proteins have consistently produced brain uptake levels of 1–3% ID per brain and high brain volumes of distribution, which were validated by the capillary-depletion technique and *in vivo* experimental models of lysosomal storage disorders (LSD), stroke, Parkinson's and Alzheimer's disease, respectively.^{34,37,52,54,55,57–59} The capillary depletion method was developed for ligands that bind to the target BBB receptor with high affinity, which produces no dissociation from the capillary receptor during the homogenization process. Measurement of the enzyme activity of vascular markers, like gamma-glutamyl transpeptidase and alkaline phosphatase, showed that the post-vascular supernatant was 94–95% depleted of brain vasculature.⁶

Clinical trials are in progress with high affinity TfRMAB fusion proteins, both in the conventional bivalent format and in a monovalent high affinity construct design, *i.e.*: pabinafusp alfa and lepunafusp alfa for Hunter's MPSII and Hurler's MPSI, respectively.^{33,34} Pabinafusp alfa was the first brain



penetrating bivalent and high affinity TfR biotherapeutic approved by a regulatory agency for the treatment of Hunter's MPSII syndrome.³⁶ Low affinity monovalent constructs targeting the TfR continue to be developed as well, and clinical trials are in progress with trontinemab for Alzheimer's disease and tividenufusp alpha for Hunter's MPSII.^{35,53} Recent publications aimed to fine-tune the affinity of TfRMAB for the BBB-TfR using the mouse specific 8D3 MAb.^{68–70}

The brain uptake through BBB receptor-mediated transport systems is a function of 3 components, (i) the antibody affinity for the receptor, (ii) the injection dose, and (iii) the plasma area under the curve (AUC). The latter may be affected by the therapeutic domain of a fusion protein. For example, when IDUA is fused to a brain penetrating transport MAb, the fusion protein shows reduced AUC compared to the MAb alone, due to increased uptake by peripheral mannose-6-phosphate (M6P) receptors.⁷¹ Kinetics modelling of TfR and/or HIR receptor-mediated transport across the BBB with either TfRMAB or HIRMAb showed that the optimal receptor-binding would be a MAb with a K_D of 0.5–5 nM and with an association rate constant (k_{on}) of 10^5 – 10^6 $M^{-1} s^{-1}$, producing a dissociation $T_{1/2}$ of ~10–120 min.⁷² Furthermore, the kinetics modelling also showed that the lower the affinity of the antibody for the TfR, the greater the ID required to maintain a given brain AUC.⁷² For example, the brain AUC of a TfRMAB-IDUA fusion protein with a moderate affinity for the TfR, $K_D = 36$ nM, would require an injected dose of 30 mg per kg BW to produce a brain AUC comparable to the one of a TfRMAB-IDUA fusion protein with high affinity ($K_D = 0.36$ – 3.6 nM) at a 10-fold lower injected dose of 3 mg per kg BW.⁷¹ A lower therapeutic dose may also be preferred to reduce potential adverse effects, as in the case of IgG-neurotrophic factor fusion proteins.^{46,73}

3. Engineering and efficacy of brain penetrating biotherapeutics

3.1. Chemical conjugates of brain penetrating biotherapeutics

The demonstration that MAbs directed to the BBB-TfR are able to produce pharmacological effects in brain were reported in the early 1990s. Using the OX-26 MAb directed to the rTfR conjugated to streptavidin (SA), central nervous system (CNS) pharmacological effects were achieved after systemic administration of biotinylated peptides, *i.e.*: nerve growth factor or vasopressin intestinal peptide.^{20,21} The application of this brain delivery system has also been extended to antisense oligonucleotides (ASO).^{74–77} The imaging of a brain tumour expressing the luciferase gene was reported with an anti-luciferase biotinyl- $[^{125}I]$ -peptide nucleic acid (PNA) bound to OX-26-SA (Fig. 2). On the contrary, both anti-luciferase biotinyl- $[^{125}I]$ -PNA alone and an unrelated biotinyl- $[^{125}I]$ -PNA control produced no imaging of the brain tumour (Fig. 2).⁷⁵ The construction and efficacy of chemically conjugates targeting either the transferrin or the insulin receptor in mouse and non-human primates have also been reported, including

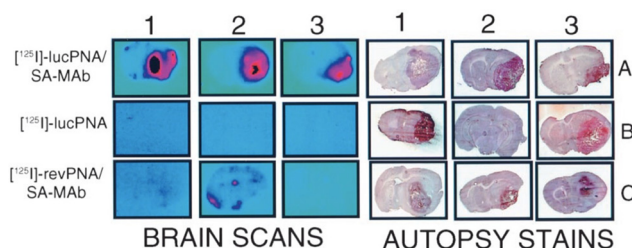


Fig. 2 Brain scans (left) and autopsy stains (right) are shown for three groups of rats designated A, B, and C. Group A rats received an IV injection of the $[^{125}I]$ -anti-luciferase PNA bound to the conjugate of the OX26 TfRMAB-SA, which is designated SA-MAB. Group B rats received $[^{125}I]$ -anti-luciferase PNA without conjugation to OX26 TfRMAB-SA. Group C rats received an IV injection of $[^{125}I]$ -anti-rev PNA bound to the OX26 TfRMAB-SA conjugate, which is a negative control. From ref. 75.

siRNA.^{21–25,60} The interest for developing delivery systems for ASO by targeting the TfR has recently been revisited and publications reported chemical conjugations of ASO to anti-TfR-Fab fragment and another TfR binding construct.^{78,79}

3.2. Non-viral gene delivery

The discovery of brain-penetrating receptor-specific MAbs, such as HIRMAb and TfRMAB, also enabled the design of Trojan horse liposomes or immune-liposomes (IML) aimed for the non-viral, non-invasive delivery of plasmid DNA to the brain.^{80–86} IMLs are pegylated liposomes containing a supercoiled plasmid DNA molecule in the interior of the liposome (Fig. 3A). IMLs are engineered with a mixture of natural and optimized lipids for the encapsulation of plasmid DNA. The brain-penetrating MAbs, *i.e.*, the 8D3 anti-mouse TfRMAB, the OX26 anti-rat TfRMAB or 83-14 anti-human HIRMAb, are conjugated to the liposome *via* a stable thioether linkage (Fig. 3A). The structure of the IML is shown by transmission electron microscopy in Fig. 3B. Mouse IgG molecules conjugated to the tips of the polyethylene glycol strands on the surface of the IML were detected with a conjugate of 10 nm gold and an anti-mouse secondary antibody.⁸⁷ IMLs have been engineered with a single transport MAb, *i.e.*, TfRMAB or HIRMAb, or with dual transport MAbs for 3-barrier models, as in the case of U87 human brain tumour models in Severe Combined Immunodeficiency (SCID) mice, wherein the 8D3 TfRMAB carries the IML across the mouse BBB and the 83-14 HIRMAb allows for receptor mediated endocytosis and nuclear targeting in U87 glioblastoma cells (Fig. 3C).

Global expression of a β -galactosidase (lacZ) transgene is shown in all parts of the brain following intravenous injection of the lacZ reporter gene encapsulated in IML (Fig. 4). The IML was engineered with the 8D3-TfRMAB for studies in mice (Fig. 4, top) or the HIRMAb for studies in the Rhesus monkey (Fig. 4A–F).^{83,87} Gene expression was widely detected through the cortical and subcortical structures of mouse and monkey brain, with a greater gene expression in grey matter relative to white matter (Fig. 4, top, A and C). The lacZ histochemistry of control un-injected primate brain shows no β -galactosidase



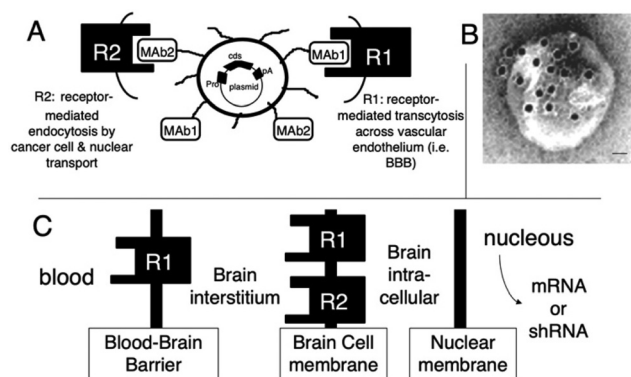


Fig. 3 Engineering of immune liposomes (IML). (A) A supercoiled expression plasmid DNA is encapsulated in the interior of the IML. The surface of the liposome contains several thousand strands of 2000 Da polyethylene glycol (PEG) to stabilize the complex in blood, and approximately 1–2% of the PEG strands are conjugated with a targeting receptor (R-) specific monoclonal antibody (Mab), i.e.: TfRMAB or HIRMAb. The latter triggers transport of the IML across biological barriers *in vivo*. The IML may be engineered with a single type of Mab to target both the BBB and brain cells in the same species. The IML may be constructed with two different MABs, as in the case of an experimental mouse model of a human brain tumour (A and C). Thus, the 8D3 mouse transferrin receptor (TfR) Mab (Mab1) targets the mouse BBB (C, R1) and the 83-14 human IR Mab (Mab2) target the human tumour cells (C, R2). (B) Transmission electron microscopy of a IML. The mouse IgG molecules conjugated to the tips of the PEG strands on the surface of the IML were detected with a conjugate of 10 nm gold and an anti-mouse secondary antibody. The position of the gold particles illustrates the relationship of the PEG-extended Mab and the liposome surface. Magnification bar = 20 nm. (C) The multiple barrier model for gene therapy of the brain. Following IV injection, the IML carrying the transgene traverses 2 barriers in series followed by the nuclear membrane to be able to reach the nucleus for expression: (i) the blood–brain barrier, (ii) the brain cell membrane, and (iii) the nuclear membrane. Reproduced from ref. 85 with permission from Wiley, copyright 2011.

activity (Fig. 4B). Light micrographs of the primate brain show gene expression in the choroid plexus epithelium, the ependymal lining of the ventricle and the capillary endothelium of the adjacent white matter, the neurons of the occipital cortex, the granular layers of the cerebellum and the Purkinje cells (Fig. 4D–F). The ectopic expression of the lacZ with the SV40 driven vector was also observed in tissues expressing either the TfR or the IR, like the spleen (Fig. 4, top left). It is possible to engineering the transgene to incorporate tissue or organ specific promoter to avoid expression of the exogenous gene in tissues other than brain.⁸⁸ Thus, the expression of a lacZ plasmid constructed with the brain glial fibrillary acidic protein (Gfap) specific promoter restricts the expression of the lacZ gene to the brain of mice injected with 8D3 TfRMAB–IML carrying the Gfap–lacZ plasmid (Fig. 4, left top panel). The spleen of these animals was negative, as the Gfap promoter is not read (Fig. 4, right top panel). On the other hand, the expression of β -galactosidase was detected in brain and spleen where the widely read SV40 promoter was used in the transgene (Fig. 4, left top panel). Along the same line, the expression of the transgene was restricted to the eye in pri-

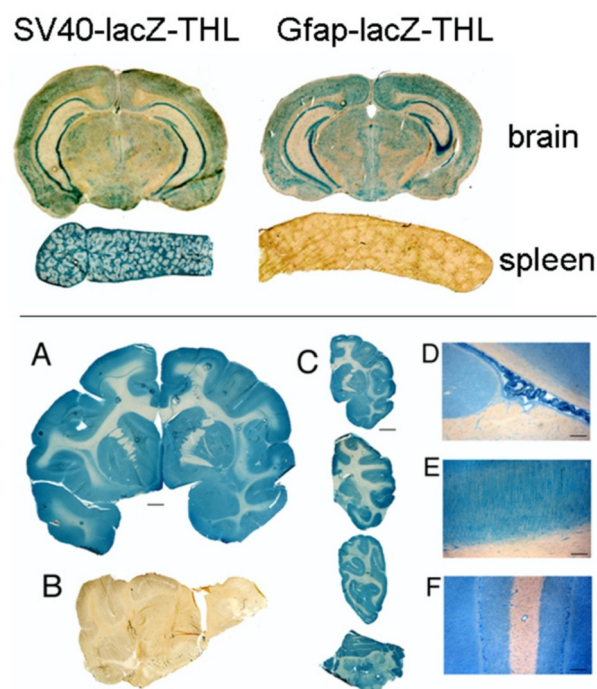


Fig. 4 *In vivo* gene expression following systemic administration of IMLs packaged with a β -galactosidase expression vector. (Top) Effect of an organ-specific promoter. IMLs were prepared with the 8D3 anti-mouse TfR and packaged with β -galactosidase expression gene driven by either the SV40 promoter (SV40–lacZ–THL) (top left) or Gfap promoter (Gfap–lacZ–THL) (top right). The transgene is expressed in the brain and spleen when the expression is directed by the SV40 promoter (top left), but just in brain when driven by the brain specific promoter, Gfap (top right). (Bottom) β -Galactosidase histochemistry of brain removed from either the HIRMAb–IML injected rhesus monkey (A, C, D, E, and F) or the control, non-injected rhesus monkey (B). The β -galactosidase expression plasmid is driven by the SV40 promoter. (A) Is a reconstruction of the two halves of a coronal section of the forebrain. (C) Shows half-coronal sections through the primate cerebrum and a full coronal section through the cerebellum; the sections from top to bottom are taken from the rostral to caudal parts of brain. (D–F) Are light micrographs of choroid plexus, occipital cortex, and cerebellum, respectively. All specimens are β -galactosidase histochemistry without counter-staining. The magnification in A and B is the same (magnification bar = 3 mm); the magnification bar in C is 8 mm; the magnification bars in D–F are 155 mm. Reproduced from ref. 85 with permission from Wiley, copyright 2011.

mates using IML carrying the lacZ gene under the opsin promoter.⁸⁹

The IML technology was also used in mouse and rat models of neural disease. For example, for the treatment of an experimental human brain cancer in SCID mice, a plasmid DNA encoding a short hairpin RNA (shRNA) against the epidermal growth factor receptor (EGFR) mRNA was encapsulated in IML and targeted to the U87 human cancer implanted in the mouse brain with combined HIRMAb and TfRMAB transport MABs (Fig. 3A and C).^{90,91} The mouse TfRMAB allowed for the transport of the IML across the mouse BBB, and the HIRMAb enable penetration into the human tumour implanted in the mouse brain. Weekly treatment of mice with intra-cranial U87



brain cancer with the MAb-targeted IMLs resulted in a 100% increase in survival time (Fig. 5).⁹¹ Therapeutic levels of a lysosomal enzyme, glucuronidase (GUSB), were also achieved in GUSB null mice, a model of type VII mucopolysaccharidosis, with TfRMAB-IML delivering a GUSB expression plasmid to the brain.⁹² IMLs were also applied to a mouse model of Niemann-Pick C1.⁹³ The IML technology was also successfully applied to an experimental rat model of Parkinson's disease with intravenous treatment with IMLs carrying a plasmid DNA that encoded for tyrosine hydroxylase (TH).^{94–96} The expression of the TH transgene was confined to the striatum by placement of the TH gene under the influence of the tyrosine hydroxylase gene promoter, showing increased expression of striatum TH and marked reduction in the PD's symptoms.⁹⁶ The average size of IML approximated 80 nm in diameter,⁸⁷ suggesting that the transport across the BBB by targeting these RMT systems may be applicable to other large molecules, like viral particles. AAV particles have been modified to target the BBB-TfR, demonstrating their potential for viral gene delivery to the brain.^{97,98}

3.3. Brain penetrating IgG-fusion proteins

The field of brain-penetrating IgG fusion proteins has attracted attention from academia and the biotech industry, with numerous validated compounds (Tables 1 and 2) and both completed and ongoing clinical trials.^{33–35,53}

3.3.1. Treatment of Hurler's syndrome (MPSI) with brain penetrating IgG-iduronidase (IDUA). Valanafusp alpha is a fusion protein comprised of HIRMAb and the human IDUA, enzyme mutated in MPSI,⁹⁹ and it was the first brain penetrat-

ing biotherapy completing a phase I/II clinical trials in Hurler MPSI.³² The HIRMAb-IDUA, designated AGT-181,¹⁰⁰ was engineered by fusion of the human mature IDUA to the C-terminus of the heavy chain of the full HIRMAb *via* a short Ser² linker.¹⁰¹ The structure of the HIRMAb-IDUA is similar to the fusion protein shown in Fig. 6 (left), with the exception that a short linker was used in lieu of an extended one. This design positions the IDUA enzyme in a dimeric configuration. HIRMAb-IDUA showed high affinity for the HIR (*i.e.*: ED₅₀ = 0.93 nM) and maintained a specific enzyme activity comparable to the mature recombinant IDUA.¹⁰¹ The bifunctionality of the HIRMAb-IDUA was validated in Hurler fibroblasts, as significantly reduced the accumulation of glycosaminoglycans (GAG) in the lysosomal compartment.¹⁰¹ *In vivo* biodistribution in the Rhesus monkey showed a global distribution throughout the brain with a volume of distribution (VD) of >700 $\mu\text{L g}^{-1}$ brain (Fig. 7).⁶² On the contrary, recombinant IDUA's brain VD of 14 $\mu\text{L g}^{-1}$ represents the blood volume in brain.⁶² The IDUA VD in brain is comparable to the one of a non-specific IgG, which is not transported across the BBB.^{38,62} The VD of HIRMAb-IDUA in the brain parenchyma, *i.e.*: post-vascular supernatant, was 3-fold higher than the VD in the brain microvasculature, demonstrating that most of the fusion protein penetrated the BBB.⁶² The peripheral organ distribution of the HIRMAb-IDUA was similar to the distribution of recombinant IDUA, as both are taken up in the peripheral organs through the M6P receptor, but with increased uptake in bone marrow and vertebral bodies (Fig. 7).⁶² The brain uptake of the HIRMAb-IDUA was reported to be 1.2% ID per brain.⁶² The levels of normal enzyme activity of IDUA in a human brain approximates 0.5–1.5 units per mg of protein.¹⁰² Therefore, it may be possible to normalize the levels brain IDUA in a Hurler individual with the administration of 1 mg per kg BW of the fusion protein, which may produce a brain concentration of 3.0 ng mg^{-1} of brain protein, which is equivalent to 1.1 units of IDUA enzyme activity per mg of brain protein.⁶² This hypothesis is supported by a study in a mouse

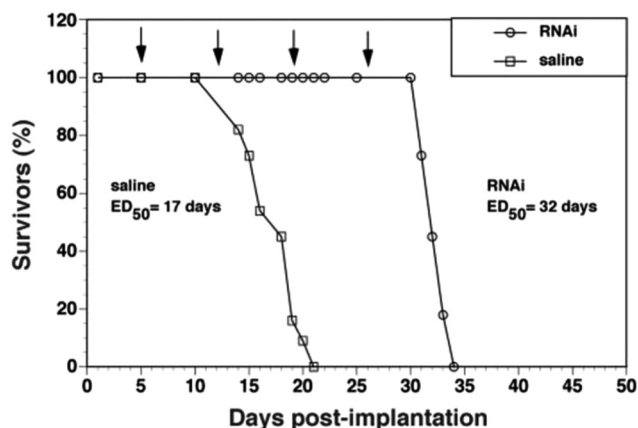


Fig. 5 Survival study following *in vivo* intravenous brain cancer RNAi gene therapy with IMLs and an anti-human EGFR shRNA plasmid. Intravenous RNAi gene therapy directed at the human EGFR is initiated at 5 days after implantation of 500 000 U87 cells in the caudate putamen nucleus of SCID mice, and weekly intravenous gene therapy is repeated at days 12, 19, and 26 (arrows). The control group was treated with saline on the same days. The time at which 50% of the mice were dead (ED₅₀) is 17 days and 32 days in the saline and RNAi groups, respectively. The RNAi gene therapy produces an 88% increase in survival time, which is significant at the $p < 0.005$ level (Fisher's exact test, $n = 11$ mice in each group). Reproduced from ref. 91 with permission from American Association for Cancer Research, copyright 2004.

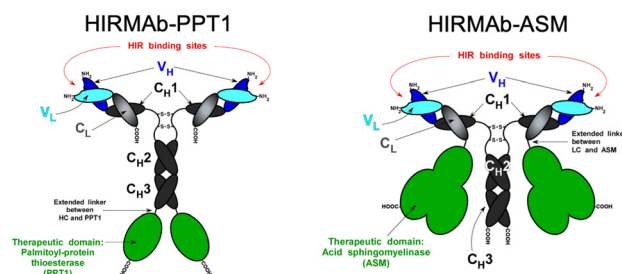


Fig. 6 Examples of genetic engineering of IgG fusion proteins with full anti-human insulin receptor MAb as BBB transport domain for the treatment of lysosomal storage disorders. The therapeutic domain of the IgG bifunctional fusion protein is fused to the C-terminus of either the heavy (left) or light chain (right) of the transport monoclonal antibody, *via* an extended linker or a short linker (not shown). The indication for these IgG fusion proteins is: HIRMAb-palmitoyl-protein thioesterase (PPT1), batten disease type 1; and HIRMAb-acid shingomyelinase (ASM), Niemann-Pick disease types A and B. From ref. 43.



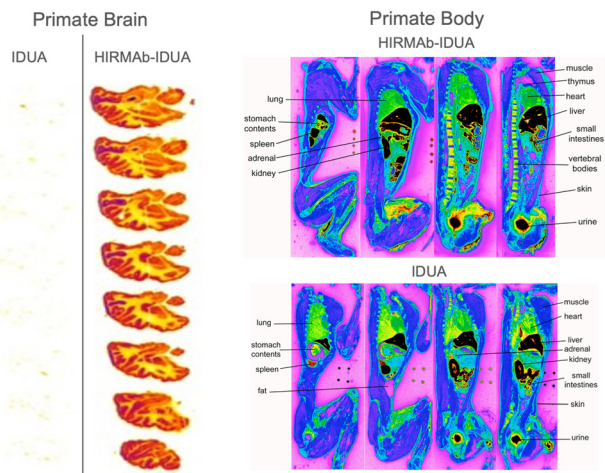


Fig. 7 (Left) Autoradiography through eight parallel sagittal sections of the cerebral hemisphere of the Rhesus monkey obtained 2 h after the IV administration of either the [125 I]-HIRMAb-IDUA fusion protein (valanafusp alpha, AGT-181) or [125 I]-IDUA. The section on the bottom is the most lateral part of brain, and the section on the top is the most medial part of brain. The cerebellum is visible in the more medial sections of the brain. The BBB-penetrating HIRMAb-IDUA produces a global distribution throughout this organ. IDUA does not cross the BBB, showing just background activity in the primate brain. The peripheral organ distribution of the HIRMAb-IDUA is similar to the distribution of recombinant IDUA, as both are taken up in the peripheral organs through the M6P receptor, but with increased uptake of HIRMAb-IDUA in bone marrow and vertebral bodies. Reproduced from ref. 62 with permission from American Chemical Society, copyright 2017.

model of MPSI with a subrogate fusion protein targeting the mouse TfR and fused to the mouse IDUA.⁵¹ The anti-mouse TfRMAB was based on a chimeric MAb comprised of the rat anti-mouse 8D3 MAb variable region and mouse IgG1 and Kappa constant region, respectively.³⁰ The anti-mouse TfRMAB-IDUA showed both high affinity for the mouse TfR ($K_D = 1.8$ nM) and high IDUA enzyme activity (776 units per μ g protein). The administration of 1 mg per kg BW mouse TfRMAB-IDUA IV twice weekly for 8 weeks produced a marked reduction in brain lysosomal inclusion bodies.⁵¹ In addition, the treatment with mouse TfRMAB-mIDUA markedly reduced the levels of GAG in the peripheral organs, which were comparable to the reported data with recombinant IDUA.⁵¹

Phase I/II clinical trials in paediatric Hurler MPSI were completed with valanafusp alpha (AGT-181) in patients previously treated with laronidase (NCT02371226, NCT02597114, NCT03053089, NCT03071341).³² Patients were treated with weekly IV infusions of valanafusp alpha at 1, 3, or 6 mg kg⁻¹ for up to 12 months. A dramatic improvement in somatic parameters was reported following treatment with valanafusp alpha for 52 weeks, *i.e.*: 23% and 26% reductions in liver and spleen volumes, respectively, and compared with the respective baseline levels.³² The improvement in the somatic parameters was attributed to the dual targeting of valanafusp alpha in the peripheral organs *via* both the insulin and M6P receptor, respectively. There was a significant increase in shoulder

flexion and extension following treatment with valanafusp alpha for 26 weeks, probably related to the preferential targeting of the HIRMAb-IDUA to vertebral bodies and joints observed in primates (Fig. 7).⁶² A mean improvement across all cognitive domains was also reported in both severe and attenuated MPSI patients treated with valanafusp alpha.³²

Lepunafusp alfa (JR-171) is a new brain penetrating fusion protein for the treatment of Hurler's syndrome MPSI (Table 2).^{33,103} In lepunafusp alfa, the transport domain is a Fab that binds with high affinity to the human TfR ($K_D = 1.88$ nM). Its therapeutic domain is the mature human IDUA fused to the C-terminus of the VH and CH1 of the humanized anti TfR-Fab *via* a (GGGGS)₃ linker.¹⁰³ The IV administration of JR-171 at 1 mg per kg BW to MPSI mice markedly reduced the concentrations of dermatan sulfate (DS) and heparan sulfate (HS) in the central nervous system and peripheral tissues. The peripheral effects of JR-171 were similar to ones seen with conventional ERT with IDUA. In addition, it was reported that JR-171 improved spatial learning ability.¹⁰³ Following its pre-clinical validation, Lepunafusp alfa completed a phase I/II clinical trial in Hurler's MPSI syndrome (NCT04227600)³³ with an extension study in progress (NCT04453085). Patients were dosed with escalating weekly IV infusions of lepunafusp alfa at 1.0, 2.0, and 4.0 mg per kg BW for 12 weeks. It was reported that lepunafusp alfa was safe and well tolerated. The somatic efficacy lepunafusp alfa was demonstrated by the reductions in serum and urinary of the IDUA substrate in naive subjects, as well as in the subjects previously treated with laronidase. A decrease in the IDUA substrate concentrations in the CSF was reported, along with positive neurocognitive and behavioural changes seen in some patients.³³

3.3.2. Treatment of Hunter's syndrome (MPSII) with brain penetrating IgG-iduronate 2-sulfatase (IDS). Pabinafusp alfa (JR-141) is a brain penetrating fusion protein comprised of a full MAb directed to the hTfR and the human IDS, enzyme mutated in MPSII,¹⁰⁴ and represents the first brain penetrating biotherapeutic approved by a regulatory agency for the treatment of Hunter MPSII syndrome.³⁵ Pabinafusp alfa was engineered as described above for valanafusp alpha/AGT-181, wherein the mature human IDS is fused to the C-terminus of the heavy chain of an anti-human TfR MAb *via* a short SS linker, which results in a bivalent fusion protein with the IDS in dimeric configuration.¹⁰⁵ The fusion protein retained high affinity for the human TfR ($K_D = 1.22$ nM) with a specific activity comparable to the recombinant IDS.¹⁰⁵ Following the IV injection of 1 mg per kg BW, pabinafusp alfa was detected in the brain of a mouse model of MPS II, *i.e.*: hTfR knockin mice lacking IDS. It also reduced the accumulation of GAGs both in the peripheral tissues and in the brain.¹⁰⁵ Another mouse specific TfRMAB fused to the human IDS has been reported and showed high brain uptake, *i.e.*: 1.3% ID per mouse brain.⁵² Phase I/II clinical trials in Hunter MPSII patients with pabinafusp alfa were completed with weekly dose escalation up to 2 mg per kg BW for a total of 4 weeks (NCT03128593, NCT03359213).¹⁰⁶ Mild adverse reactions were reported with reduction in the levels of heparan sulfate in



plasma, urine and CSF.¹⁰⁶ A phase II/III trial was also completed with pabinafusp alfa in MPSII, at a weekly dose of 2 mg per kg BW IV for 52 weeks (NCT03568175).³⁴ The brain penetrating IDS fusion protein produced a significant reduction in the levels of HS in CSF, which was used as the primary efficacy endpoint.³⁴ An extended phase II/III clinical trial with pabinafusp alfa in Hunter's syndrome is in progress (NCT04573023).

Another brain penetrating fusion protein for Hunter MPSII has been engineered and well characterized, HIRMAb-IDS (AGT-182). This fusion protein has been designed using a similar strategy as the one described for HIRMAb-IDUA (AGT-181) in section 3.3.1. (Table 1).³⁸ The cDNA of the mature human IDS was fused to the C-terminus of the heavy chain of the HIRMAb *via* a short Ser² linker.^{107,108} The HIRMAb-IDS fusion protein retained both high affinity for the HIR (ED₅₀ = 0.4 nM) and comparable IDS enzyme activity to the recombinant IDS.^{107,108} The HIRMAb-IDS fusion protein was localized to the lysosomes and markedly reduced the accumulation of GAG in Hunter fibroblasts.¹⁰⁸ The biodistributions of the HIRMAb-IDS fusion protein in the Rhesus monkey showed global brain penetration similar to the one of valanafusp alpha (Fig. 7) with a brain uptake of approximated 1% ID per brain and a brain VD of >800 $\mu\text{L g}^{-1}$ brain.³⁸ On the other hand, IDS (Elaprase) showed background activity in brain with a brain VD similar to the brain blood volume.³⁸ The uptake ratio of HIRMAb-IDS : Elaprase in peripheral organs approximated 1 as both proteins are taken up *via* the M6P receptor.³⁸ Based on the primate brain uptake of HIRMAb-IDS, a dose of 1 mg per kg BW in humans may produce brain therapeutic levels of 0.34 U mg^{-1} .³⁸ A phase I safety and dose ranging study was completed with the HIRMAb-IDS fusion protein (AGT-182) in patients with Hunter's MPSII syndrome (NCT02262338), but no results have been published.

A third construct for the potential treatment of Hunter's MPSII syndrome was reported.³⁹ This is a monovalent Fab fragment engineered to target the human IR and fused to human IDS *via* a LSS short linker, it is designated HIR-Fab-IDS (GNR-055).³⁹ This fusion protein maintained high affinity for the HIR (*i.e.*: K_D = 0.136 nM) and IDS activity similar to the recombinant IDS.³⁹ The HIR-Fab-IDS rapidly penetrated the BBB producing a brain uptake of 1% ID per brain 2 h following the IV administration of [¹²⁵I]-HIR-Fab-IDS labeled using the Bolton-Hunter reagent in cynomolgus primates.³⁹ The whole-body autoradiography showed an image comparable to the one obtained with HIRMAb-IDUA fusion protein (valanafusp alpha, AGT-181) in Fig. 7, with similar peripheral organ biodistribution of HIR-Fab-IDS and IDS, but with global distribution throughout the brain for HIR-Fab-IDS and background activity for IDS alone.³⁹ A phase II/III clinical trial in Hunter MPSII patients with HIR-Fab-IDS (GNR-055) is also in progress (NCT05208281).

Tvidenofusp alfa (ETV : IDS, DNL310) is another brain penetrating form of IDS for the potential treatment of Hunter's MPSII syndrome.⁵³ In ETV : IDS the human IDS pro-protein is fused *via* a short flexible G4S linker with a human IgG1 hinge-CH₂-CH₃ Fc fragment-bisdisulfide with a human

IgG1 hinge-CH₂-CH₃ Fc fragment engineered for binding to the human TfR1 (Fig. 8). The ETV : IDS heterodimer retains IDS enzyme activity and binds to the human TfR1 with very low affinity (*i.e.*: K_D = 150–300 nM).¹⁰⁹ The brain uptake in mice of the ETV : IDS was reported to be 9 nM at 8 h following the administration of 1 or 5 mg per kg BW, IV or IP, respectively.⁵³ A marked reduction of brain and peripheral organs GAG was also reported with weekly IP injection of 3 mg per kg BW ETV : IDS for 17 weeks.⁵³ Phase I/II (NCT04251026) and II/III (NCT05371613) clinical trials in Hunter MPSII subjects with tvidenofusp alfa are in progress.

3.3.3. Treatment of San Filippo syndrome type A (MPSIIIA) with brain penetrating IgG-N-sulfolglucosamine sulfohydrolase (SGSH). A brain penetrating fusion protein with HIRMAb fused to the human SGSH, the enzyme mutated in MPSIIIA,¹¹⁰ was engineered for the potential treatment of Sanfilippo MPSIIIA.⁴¹ The genetic engineering of the HIRMAb-SGSH fusion protein was completed as described above for other HIRMAb-enzyme fusion proteins,^{32,38} with the exception that the mature human SGSH was fused at the C-terminus of the heavy chain of the HIRMAb *via* a 31-amino acid linker corresponding to the hinge region of human IgG3, in lieu of a short Ser linker.⁴¹ This was needed to correct suboptimal expression levels in cultured cells.⁴¹ The HIRMAb-SGSH fusion protein maintained high affinity for the targeting insulin receptor (*i.e.*: EC₅₀ = 0.33 nM), and a SGSH specific enzyme activity like recombinant SGSH.⁴¹ The brain uptake in the Rhesus monkey approximated of 1% ID per primate brain, with a brain VD of >700 $\mu\text{L g}^{-1}$.⁴¹ The brain post-vascular VD was near 700 $\mu\text{L g}^{-1}$ confirming that most of the fusion protein was transported across the BBB and not retained by brain microvasculature, which showed a VD comparable to the blood volume in brain (24 $\mu\text{L g}^{-1}$).⁴¹ Based on the brain uptake in the primate, a dose of 3 mg kg^{-1} HIRMAb-SGSH may produce brain levels of 0.25 U g^{-1} ,⁴¹ which is comparable to the endogenous normal levels of SGSH in brain,¹¹¹ suggesting that it is possible to achieve

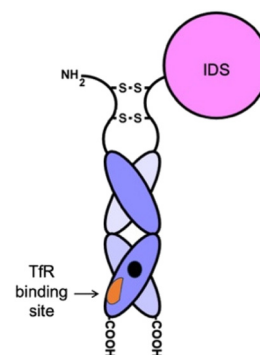


Fig. 8 Tvidenofusp alfa (ETV : IDS, DNL310) is a brain penetrating form of IDS for MPSII syndrome.⁵³ The human IDS pro-protein is fused *via* a short flexible G4S linker with a human IgG1 hinge-CH₂-CH₃ Fc fragment-bisdisulfide with a human IgG1 hinge-CH₂-CH₃ Fc fragment engineered for binding to the human TfR1. The heterodimer is engineered with the knobs-into-holes technology (black circle). Recreated from ref. 53.



therapeutic levels of SGSH with the administration of HIRMAb-SGSH fusion protein in MPSIIIA. The efficacy of the brain penetrating IgG-SGSH fusion protein was demonstrated in a mouse model of MPSIIIA with a fusion protein targeting the mouse TfR fused to the human SGSH (Table 2).⁵⁴ Two weeks old MPSIIIA mice were treated with 5 mg kg⁻¹ of the high affinity mouse TfRMAb-SGSH fusion protein IP 3× per week for 6 weeks.⁵⁴ Fig. 9 shows the heparan sulfate (HS) levels in brain and liver determined by LC-MS. The saline control MPSIIIA mice showed a 30- and 36-fold elevation in HS in brain and liver, respectively, as compared to the wild type animals (Fig. 9). The treatment with the TfRMAb-SGSH markedly reduced the levels of HS in brain (70%) and liver (85%), with no effect with the isotype control (Fig. 9).⁵⁴

A brain penetrating IgG-SGSH fusion protein, Postnafusp alfa (JR-441), has entered a phase I/II clinical trial in Sanfilippo MPSIIIA (NCT06095388) (<https://ssl4.eir-parts.net/doc/4552/tdnet/2496993/00.pdf>). The structure of this fusion protein is similar to the above described lepunafusp alfa, wherein the transport domain is a high affinity MAb directed to the human TfR in Fab format fused to mature SGSH *via* a (GGGGS)₃ linker (<https://gsrs.ncats.nih.gov>).

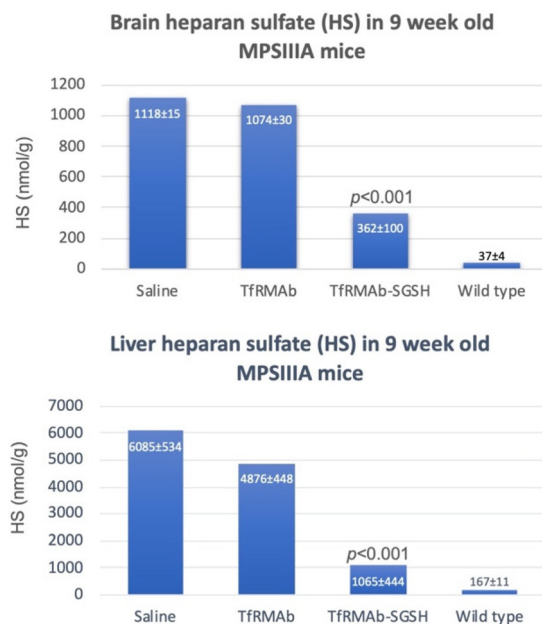


Fig. 9 Reduction in brain heparan sulfate (HS) in the MPSIIIA mouse with the administration of a mouse TfRMAb-SGSH fusion protein. Two-week-old MPSIIIA mice were treated three times weekly for 6 weeks with IP 5 mg kg⁻¹ of the TfRMAb-SGSH fusion protein or the isotype control (TfRMAb). The mice were euthanized 1 week after the last dose. Following enzymatic digestion into disaccharides, HS was measured in brain and liver by LC-MS using HS disaccharide standards. There was a 30-fold increase in HS in brain of the MPSIIIA mice as compared with the wild-type controls. The elevated HS in brain was reduced 70% by the chronic treatment with the brain penetrating TfRMAb-SGSH fusion protein (top). The HS was also elevated in liver, and treatment with the mouse TfRMAb-SGSH reduced the hepatic HS by 85% (bottom). Data are expressed as means ± SD (*n* = 8 mice/group). Reproduced from ref. 37 with permission from MDPI, copyright 2022.

Another candidate for the potential treatment of MPSIIIA, EVT:SGSH (DNL126), was announced to begin an open-label phase I/II study in Sanfilippo MPS IIIA subjects (NCT06181136). The structure of EVT:SGSH is similar to the one EVT:IDS (tividenofusp alfa) described above.

3.3.4. Treatment of neuronal ceroid lipofuscinosis type 1 (CLN1 disease) with brain penetrating IgG-palmitoyl protein thioesterase-1 (PPT1). A brain penetrating form of PPT1 was engineered for the potential treatment of Batten Type 1 disease or CLN1 disease by fusion of PPT1, enzyme mutated in this LSD,¹¹² to the C-terminus of the HIRMAb heavy chain (Fig. 6).⁴³ The PPT1 was linked to the HIRMAb *via* the flexible 31 amino acid extended linker discussed above.^{41,43} The fusion protein maintained high PPT1 enzyme activity and high affinity for the insulin receptor (ED₅₀ = 0.38 nM).⁴³ Brain therapeutics levels of 2.6 U may be reached with the administration of 3 mg per kg BW of the HIRMAb-PPT1, assuming a brain uptake of 1% ID.⁴³ These brain levels represent 2.2% of normal endogenous PPT1 activity; however, it would still be therapeutic as just 0.5% of endogenous PPT1 activity is needed to reverse the neuropathology of CLN1.¹¹³ Based on this calculation and on the safety obtained in the phase I/II clinical trial with valanafust alfa,³² a child with CLN1 disease was dosed with HIRMAb-PPT1, designated AGT-194, under compassionate use in Germany.¹¹⁴ AGT-194 was dosed weekly for 26 months at 2.6 mg per kg BW, and an improvement of life quality related to ameliorated control of epilepsy was reported in the absence of adverse effects.¹¹⁴

3.3.5. Treatment of other lysosomal storage disorders with brain penetrating IgG-fusion proteins. A potential treatment of metachromatic leukodystrophy (MLD) may be achieved with a brain penetrating form of Arylsulfatase A (ASA), lysosomal enzyme that is mutated in MLD.¹¹⁵ The genetic engineering of a HIRMAb-ASA fusion protein was reported, wherein cDNA of the mature human ASA was fused at the C-terminus of the heavy chain of the HIRMAb *via* a short Ser-Ser linker (Table 1).⁴⁰ The HIRMAb-ASA fusion protein retained high ASA enzyme activity and affinity for the targeting insulin receptor (ED₅₀ = 0.34 nM).⁴⁰ The ASA fusion protein was triaged to the lysosomal compartment and produced a global distribution throughout the brain, with a brain uptake of 1.1% ID per primate brain, and a brain VD of 526 μL g⁻¹.⁴⁰ Based on the primate brain uptake, a dose 2.5 mg kg⁻¹ in humans may produce 14 ng mg⁻¹ brain protein.⁴⁰ This represents 14% of the levels of ASA in normal human brain,¹¹⁶ and it may be sufficient to treat this lysosomal storage disorder, as the replacement of just 1–2% of normal enzyme activity is expected to be therapeutic.¹¹⁷ This is supported by the finding that 5–20% of the population has ASA pseudo-deficiency, with 3–8% of normal levels of ASA enzyme activity in brain and no symptoms of MLD.¹¹⁸

The Sanfilippo type B syndrome or MPSIIIB is caused by the mutation of the α-N-acetylglucosaminidase (NAGLU) gene,¹¹⁹ and it may be treatable with a brain penetrating IgG-NAGLU fusion protein. The genetic engineering of the HIRMAb-NAGLU fusion protein has been reported, wherein



the NAGLU was fused to the C-terminus of the HIRMAb heavy chain with a 31 amino acid extended linker.⁴² The HIRMAb–NAGLU fusion protein maintained a NAGLU enzyme activity comparable to the one of the recombinant NAGLU, and high affinity for the HIR ($ED_{50} = 0.44$ nM).⁴² The HIRMAb–NAGLU fusion protein markedly reduced the levels of GAG in human MPSIIIB fibroblasts.⁴² The brain uptake in the Rhesus monkey was 1% ID per brain with a brain VD of $337 \mu\text{L g}^{-1}$.⁴² The brain VD in post-vascular fraction approximated the one of the brain homogenate, demonstrating that virtually 100% of the HIRMAb–NAGLU fusion protein was transported across the BBB and not retained in the brain microvasculature.⁴² With this level of brain uptake, the administration of 1 mg per kg BW may produce 0.36 U NAGLU per mg brain protein, which is comparable to the NAGLU enzyme activity in normal brain.¹²⁰

A brain penetrating IgG–NAGLU fusion protein, JR-446, has entered a phase I/II clinical trial in Sanfilippo MPSIIIA (NCT06488924). In this fusion protein, the transport MAb is of a high affinity directed to the human TfR and fused to NAGLU (<https://curesanfilippofoundation.org/2024/07/jr-446-enzyme-replacement-mps-iiib-phase-i-ii/>).

The mutation of the acid shingomyelinase (ASM) gene produces the Niemann-Pick A (NPDA) syndrome.¹²¹ Since ASM does not cross the BBB, it is possible to treat the brain in NPDA with brain penetrating IgG–ASM fusion protein. A HIRMAb–ASM fusion protein was engineered by fusion of the ASM gene to the light chain of HIRMAb via a 31 amino acid extended linker (Table 1) (Fig. 6).⁴³ This configuration is preferred as it places the ASM in monomeric configuration allowing to form a hetero dimer with saposin C,¹²² which is needed to express ASM enzymatic activity (Fig. 6).⁴³ The HIRMAb–ASM fusion protein showed high affinity for the targeting insulin receptor ($ED_{50} = 0.93$ nM), and high ASM enzyme activity.⁴³ Assuming a brain uptake for the HIRMAb–ASM fusion protein of 1% ID, an IV dose of 3 mg per kg BW of the fusion protein may produce a brain concentration of 1.5 mg per brain, which represents a therapeutic level of ASM in the brain of a NPDA mouse.¹²³

The Tay-Sachs disease is produced by the mutation of the Hexosaminidase A (HEXA).¹²⁴ A HIRMAb–HEXA fusion protein was designed as described above for ASM as potential treatment for Tay-Sachs disease.⁴³ The HEXA gene was fused to the light chain of HIRMAb to place this enzyme in monomeric configuration (Fig. 6) (Table 1).⁴³ The HIRMAb–HEXA is able to form a hetero-dimer complex with the GM2 activator protein.¹²⁵ The HIRMAb–HEXA fusion protein showed high affinity for the targeting insulin receptor ($ED_{50} = 0.35$ nM) and high HEXA enzyme activity comparable to the one of the recombinant HEXA protein.⁴³ Assuming a brain uptake of 1% ID and an IV dosing of 3 mg per kg BW HIRMAb–HEXA, a brain concentration of 2.5 U per brain may be obtained, which represents a therapeutic enzyme level in the Tay-Sachs brain.¹²⁶

The gene mutated in GM1-gangliosidosis is the β -galactosidase (GLB1).¹²⁷ A brain penetrating form of GLB1

was produced by fusion of the mature human GLB1 to the C-terminus of the heavy chain of the HIRMAb with a flexible 31 amino acid extended linker (Table 1), as shown in Fig. 6 for HIRMAb–PPT1.⁴³ This fusion protein had high affinity for the insulin receptor ($ED_{50} = 0.36$ nM) maintaining the GLB1 enzyme activity.⁴³ As discussed above for other brain penetrating fusion enzymes, if a brain uptake of 1% ID is assumed, the IV administration of 3 mg per kg BW of the HIRMAb–GLB1 fusion protein may produce a brain concentration of 256 U g^{-1} brain, and this represents a therapeutic GLB1 enzyme level in the GLB1 brain.¹²⁸ The engineering of a mouse TfRMAb–GLB1 fusion protein was also reported. This fusion protein was able to increase the GLB1 enzyme activity in the mouse liver by 20%, however, it failed to increase the GLB1 activity in the brain or reduce ganglioside content.¹²⁹ This fusion protein was engineered with a suboptimal short serine linker known to produce low levels of GLB1 activity, as opposed to the 31 amino acid extended linker reported for this fusion protein.⁴³ Additional studies with a fusion protein with optimal GLB1 enzyme activity may be needed to clarify this matter.

3.3.6. Treatment of neurodegeneration with brain penetrating IgG-fusion proteins. Several brain penetrating IgG-fusion proteins have been developed for the potential treatment of neurodegenerative diseases, like Alzheimer's and Parkinson's disease, respectively, and those include bispecific MAbs, decoy receptors and neurotrophic factors.

3.3.6.1. Treatment of Alzheimer's disease with brain penetrating bispecific MAb fusion proteins. Even though conventional MAbs have been approved for anti-amyloid immunotherapies of Alzheimer's disease (AD),¹³⁰ serious adverse effects were associated with their use, *i.e.*: Amyloid Related Imaging Abnormalities (ARIA), including vasogenic oedema and cerebral microhaemorrhages.¹³¹ Aimed to increase brain uptake and to reduce adverse side effect, a series of brain penetrating bispecific MAb have been engineered.^{35,44,55,56,132} An anti-AD A β peptide in a scFv configuration was fused to the C-terminus of the transport HIRMAb to form a tetravalent bispecific HIRMAb–A β (Fig. 10, left).^{44,133} A second version of this tetravalent bispecific MAb targeting the mouse BBB, TfRMAb–A β , has also been reported,⁵⁵ wherein the transport domain is a chimeric MAb based on the 8D3 anti-mouse TfR sequence.³⁰ These anti-A β MAbs were engineered to cross the BBB in both directions for immune therapy of AD.¹³³ This process involves the transport of the anti-A β antibody across the BBB, binding to and disaggregation of A β fibrils in the brain and the efflux of the anti-A β antibody from the brain back into the blood. All three steps have been demonstrated for the HIRMAb–A β .¹³³ The tetravalent bispecific HIRMAb–A β maintained high affinity for both HIR ($ED_{50} = 1.9$ nM) and A β^{1-40} ($ED_{50} = 2.0$ nM).⁴⁴ The biodistribution in the Rhesus monkey showed global distribution of the fusion MAb with a preferential uptake in grey matter relative to white matter.^{44,133} The capillary depletion technique showed large brain volume of distribution in brain homogenate, which was comparable to the post-vascular fraction, demonstrating that the HIRMAb–A β was transported across the BBB and not retained into the microvasculature.¹³³



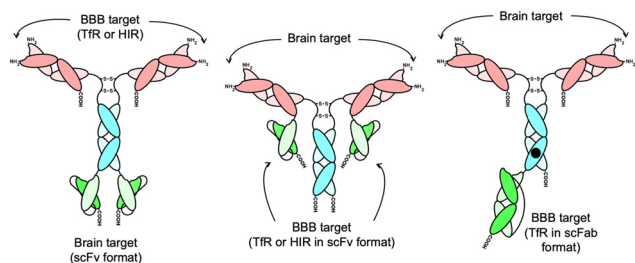


Fig. 10 Brain penetrating bispecific MABs. (Left) In this construct, the transport domain of the bispecific fusion protein is in full MAB format targeting the BBB TfR or HIR, and the therapeutic domain, *i.e.*: anti-AD Aβ peptide, in a dimeric scFv configuration.^{44,133} (Center) In this construct, the therapeutic MAB, anti-AD Aβ peptide RmAb158,¹³⁸ is maintained as full IgG, and the transport domain is the 8D3 MAB in a scFv format fused to the C-terminus of the light chain of the RmAb158.^{56,137} (Right) Trontinemab is a recombinant (2 + 1) bispecific humanized MAB anti-Aβ (gantenerumab) and binds to TfR monovalent in scFab format.³⁵ The heterodimer is engineered with the knobs-into-holes technology (black circle). Recreated from ref. 35, 44, 56 and 133.

The mouse TfRMAB-Aβ bispecific Mab fusion protein maintained high affinity for both Aβ and the mouse TfR, producing a brain uptake of 3.5% ID.⁵⁵ The administration of 1 mg per kg BW TfRMAB-Aβ IV trice weekly, or 5 mg per kg BW SC daily, for 12 weeks to PSAPP double transgenic mice produced a marked reduction in the brain concentration of Aβ1-42 (*i.e.*: 40–61%),^{134,135} without brain micro-haemorrhage, common adverse side effect seen in the immune therapy of AD.^{131,136} Another brain penetrating mouse specific tetravalent MAB based on the 8D3 sequence was engineered.^{56,137} In this construct the therapeutic MAB, a recombinant variant of mAb158 (RmAb158),¹³⁸ is maintained as full IgG, and the transport domain is the 8D3 MAB in a scFv format fused to the C-terminus of the light chain of the RmAb158 (Fig. 10, centre).^{56,137} It is claimed that even though the tetravalent RmAb158-scFv8D3 has 2 binding sites for the mouse TfR, it acts as monovalent MAB for the TfR, as both scFv8D3 are spatially separated and unable to bind 2 TfRs.^{56,137} The RmAb158-scFv8D3 has been pre-clinically validated in a mouse model of AD by both imaging and reduction of soluble Aβ protofibrils.¹³⁷ A monovalent scFv8D3 fused to Aβ-affibodies was also reported to produce global distribution throughout the brain and uptakes to up to 1.2% ID g⁻¹ brain.¹³⁹ TfR-tetravalent bispecific tandem-Aβ IgGs based on the anti-mouse 8D3 TfRMAB were engineered with affinities ranging from 2 to 29 nM and they were effective in an animal model of AD without serious adverse effects on both hematological parameters and organ histopathology after chronic treatment.¹³²

Trontinemab is the first brain penetrating biological completing a phase I/II clinical trial for the immunotherapy of AD (NCT04639050).³⁵ Trontinemab is a recombinant (2 + 1) bispecific humanized MAB anti-Aβ (gantenerumab) and binds to TfR (Fig. 10, right). The MAB anti-Aβ is fused to an anti-TfR Fab fragment *via* a GGS(GGGGS)3 linker, and it has a low

affinity for the TfR ($K_D = 131$ nM).³⁵ Preliminary data from the phase I/II clinical study showed encouraging results in subjects receiving both 1.8 mg kg⁻¹ or 3.6 mg kg⁻¹ intravenous dose of trontinemab every 4 weeks for 28 weeks. Both doses shown rapid and significant amyloid lowering in brain by amyloid positron emission tomography. AD biomarkers were also reduced in CSF, *i.e.*: including amyloid and total and phosphorylated Tau. Trontinemab also had a good safety profile, with ARIA occurring in less than 5% of participants, *i.e.*: three mild cases of ARIA-E (edema) and the five cases of ARIA-H (hemorrhage). (<https://www.alzforum.org/news/conference-cov-erage/trontinemab-fuels-hope-brain-shuttle-lift>).

Another brain penetrating antibody transport vehicle (ATV) was reported for the immunotherapy of Alzheimer's disease. This anti Ab MAB has asymmetrical Fc mutations (ATV^{cisLALA}) engineered to mitigate TfR-related hematological adverse side effects, but maintaining the ability to induce microglia phagocytosis of Aβ *ex vivo* and plaque reduction *in vivo*. In addition, this construct presented virtually complete elimination of ARIA lesions and vascular inflammation.¹⁸⁷

3.3.6.2. Treatment of Alzheimer's disease with brain penetrating IgG-decoy receptors. Other potential new therapeutics for brain disorders, including AD, are decoy receptors. Brain penetrating decoy receptors have been engineered with either the HIRMAb or the TfRMAB and the extracellular domain (ECD) of the tumor necrosis factor alpha (TNFα) receptor (TNFR).^{45,57} TNFα decoy receptor is known to suppress inflammatory reactions in non-brain tissues.^{140,141} TNFα was associated with disorders of the CNS, including neurodegeneration, stroke, depression, traumatic brain and spinal cord injury.^{142–146} Therefore, brain penetrating forms of TNFα decoy receptor may represent potential treatments for various pathologies of the CNS. Human TNFR ECD was fused to the C-terminus of the heavy chain of the HIRMAb or the TfRMAB *via* a short Ser-Ser linker (Tables 1 and 2).^{45,57} These fusion proteins maintained high affinity for their respective BBB receptor and for the TNFα.^{45,57} The brain uptake of the HIRMAb-TNFR in the Rhesus monkey was high at 3% ID and with a brain VD of 354 μL g⁻¹ (Fig. 11).⁴⁵ The capillary depletion technique showed that the majority of the HIRMAb-TNFR was transported across the BBB, as the VD in the post-vascular fraction was comparable to the one of the brain homogenate, and the VD in brain capillary pellet was similar to the blood compartment in brain, *i.e.*: 28 μL g⁻¹.⁴⁵ The latter was also comparable to the VD of the non-brain penetrating TNFR:Fc (etanercept),¹⁴⁰ *i.e.*: 13 μL g⁻¹, which is confined to the blood compartment of the brain (Fig. 11). The ratio for the organ permeability-surface area (PS) of the HIRMAb-TNFR *versus* the TNFR:Fc in the Rhesus monkey demonstrates that HIRMAb-TNFR and TNFR:Fc are similarly transported into peripheral organs, as the PS ratio approximates 1 (Fig. 11).⁴⁵ On the contrary, the PS ratio was >30 in brain, as only the HIRMAb-TNFR is transported into the primate brain (Fig. 11).⁴⁵ The therapeutics efficacy of a brain penetrating TNFR was shown in a mouse model of AD with the TfRMAB-TNFR fusion protein. A chronic treatment with TfRMAB-TNFR, but not with either



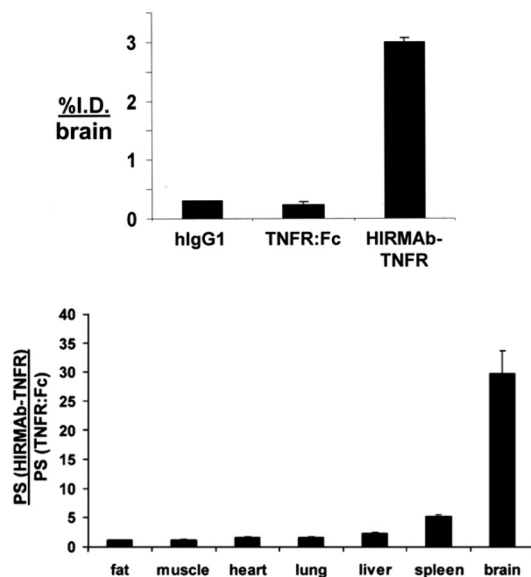


Fig. 11 Brain uptake and biodistribution of HIRMAb–TNFR and TNFR:Fc (etanercept) in the Rhesus monkey. HIRMAb–TNFR fusion protein was transported across the BBB, producing a brain uptake of 3% ID. On the other hand, the non-brain-penetrating TNFR:Fc produced a brain uptake comparable to IgG1, which are both confined to the blood compartment in the brain (top). The ratio for the permeability-surface area (PS) of the HIRMAb–TNFR relative to the PS for the TNFR:Fc in the rhesus monkey approximates 1 (bottom), as both molecules are transported into the peripheral organs. The PS ratio was >30 in the brain, as just the HIRMAb–TNFR is transported across the BBB and into the primate brain. Reproduced from ref. 37 with permission from MDPI, copyright 2022.

saline or etanercept, produced a marked reduction in neuroinflammation, and in both the A β peptide and in the plaque load, respectively, as well as in improved recognition memory.¹⁴⁷ As observed with the TfRMAB–A β -ScFv above,¹³⁴ no sign of microhaemorrhage was seen with the chronic treatment of TfRMAB–TNFR without any TfRMAB-related hematology- or iron-dysregulation in aged APP/PS1 mice.^{147–149}

3.3.6.3. Treatment of Alzheimer's disease with brain penetrating IgG-neurotrophins. Another potential treatment of AD may be developed with a brain penetrating form of erythropoietin (EPO). HIRMAb–EPO and TfRMAB–EPO were produced by insertion of the cDNA of the mature EPO at the C-terminus of the heavy chain of the transporting MAb with a short Ser–Ser–Ser linker (Tables 1 and 2).^{46,58} These fusion proteins maintained high affinity for the corresponding BBB receptor and for the EPO receptor.^{46,48} The brain uptake of HIRMAb–EPO in the Rhesus monkey was high at 2% ID per monkey brain, with a VD of 260 $\mu\text{L g}^{-1}$.⁴⁶ On the contrary, EPO does not cross the BBB and is confined to the blood compartment of the primate, showing a brain uptake comparable to non-brain penetrating human IgG1.⁴⁶ In the mouse, TfRMAB–EPO traversed the BBB with comparable brain uptake, i.e.: 2% ID per mouse brain, and a brain VD of 431 $\mu\text{L g}^{-1}$.⁵⁸ In a model of experimental AD, the mTfRMAB–EPO fusion protein presented therapeutic benefits on A β load, synaptic loss, and microglial activation, as

well as improved special memory, and without evidence of microhaemorrhage.^{150,151}

3.3.6.4. Treatment of Alzheimer's disease with brain penetrating IgG-neprilysin. A brain penetrating form of neprilysin (NEP) has been postulated as novel potential treatment for AD, as this zinc-dependent metalloprotease cleaves monomeric and pathological oligomeric A β ₄₀.^{152,153} The transport domain of the fusion protein is a monomeric Fab of the mouse anti rat TfR OX26 Mab fused to hIgG1 constant region. The extracellular domain of NEP is fused to the C-terminus of the latter. A dimer hIgG1 constant region is formed by pairing using the knobs-into-holes protocol.¹⁵³ The TfR–sFab–Fc–NEP maintained enzyme activity and the binding to the rat TfR was comparable to the high affinity dimeric OX26.¹⁵³ A brain concentration of approximately 110 ng mL^{−1} was reported 24 h after the IV administration of 10 mg per kg BW, and this was associated with a marked reduction in A β ₄₀.¹⁵³ Another brain penetrating NEP fusion protein was effective in a mouse model of AD, where the BBB transport domain was the rat anti mouse TfR 8D3 Mab in scFv format.¹⁵⁴ Along the same line, somatostatin (SST) is known to increase the activity of NEP, and an 8D3–scFv–SST fusion protein increased the brain endogenous levels of NEP and degradation of membrane-bound A β ₄₂.^{155,156}

3.3.6.5. Treatment of Parkinson's disease with brain penetrating IgG-fusion proteins. Preclinical studies have shown that brain penetrating forms of EPO, TNFR and GDNF represent potential treatments for PD.^{157,158,160} The above-described mouse TfRMAB–EPO fusion protein was neuroprotective in a 6-hydroxydopamine model of PD.¹⁵⁷ The brain penetrating TfRMAB–EPO was administered IV at 1 mg per kg BW 1 hour after the toxin and every other day for 3 weeks. The fusion protein markedly reduced apomorphine- and amphetamine-induced rotation, respectively, and increased the vibrissae-elicited forelimb placing, as well the striatal tyrosine hydroxylase (TH) enzyme activity.¹⁵⁷ The above-described TfRMAB–TNFR was also neuroprotective in the 6-hydroxydopamine mouse model of PD, reducing apomorphine- and amphetamine-induced rotation, and increasing the vibrissae-elicited forelimb placing and the striatal TH enzyme activity.¹⁵⁸ On the contrary, etanercept is not transported through the BBB and had no effect on striatal TH enzyme activity nor neurobehavior.¹⁵⁸

The genetic engineering of brain penetrating forms of GDNF have been reported targeting either the human IR or the mouse TfR, respectively (Tables 1 and 2).^{47,59,159} These fusion proteins were originally designed for the treatment of stroke; however, they may also have potential application for PD. The mature human GDNF cDNA was fused to the C-terminus of the heavy chain of the HIRMAb of the TfRMAB using the short Ser–Ser linker.^{59,159} These fusion proteins showed high affinity for both the corresponding target receptor and the GDNF receptor, respectively.^{59,159} In the Rhesus monkey, HIRMAb–GDNF showed a global distribution of the fusion protein throughout the brain, with a brain clearance (CL) of 0.8 $\mu\text{L min}^{-1} \text{g}^{-1}$.⁴⁷ On the contrary, GDNF is not transported across the BBB, and produced a brain CL comparable to non-brain penetrating human IgG1, as both molecules remain in the



blood compartment.⁴⁷ The brain uptake of the mouse TfRMAB-GDNF fusion protein was high at 3% ID per mouse brain, and with a brain VD of near 250 $\mu\text{L g}^{-1}$.⁵⁹ The TfRMAB-GDNF was also neuroprotective in the 6-hydroxydopamine mouse model of PD.¹⁶⁰ The IV administration of 1 mg per kg BW of the fusion protein 1 hour after the toxin and every other day for 3 weeks was neuroprotective reducing apomorphine- and amphetamine-induced rotation and increasing the vibrissae-elicited forelimb placing and the striatal TH enzyme activity.¹⁶⁰ A study of a GDNF fusion protein targeting the human insulin receptor was reported not to be effective in producing neuroprotection in a MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) model of PD in primates.¹⁶¹ Since the BBB-penetrating TfRMAB-GDNF was neuroprotective in the 6-hydroxydopamine mouse model of PD at the low dose of 1 mg per kg BW,¹⁶⁰ it is unclear why negative results were observed in the MPTP primate model. Besides difference in animal models, additional time course and/or dose finding studies in the MPTP model may be needed to clarify this matter.

A bispecific MAB comprised of a TrkB agonist antibody and fused to a single domain shark antibody targeting the Tfr1 was also reported to provide full neuroprotection in the 6-hydroxydopamine mouse model of PD.¹⁶²

3.3.7. Treatment of stroke with brain penetrating IgG-fusion proteins. Preclinical studies have shown that brain penetrating forms of EPO, TNFR and GDNF represent potential treatments of stroke.^{163–166} The mouse TfRMAB-EPO fusion protein was neuroprotective in a reversible middle cerebral artery occlusion (MACO) stroke model, injected at 1 mg per kg BW IV following MACO. At 24 h, TfRMAB-EPO produced a significant reduction in hemispheric stroke volume, as well as in the neuronal deficit, whereas the non-brain penetrating EPO had no effect.^{163,164} In the same MACO mouse model of stroke, TfRMAB-TNFR also produced neuroprotection causing a significant reduction in hemispheric, cortical, and subcortical stroke volumes, and neuronal deficit, respectively, whereas etanercept had no effect.¹⁶⁵ Along the same line, TfRMAB-GDNF fusion protein was neuroprotective as well in the MACO stroke model. The administration of 1 mg per kg BW IV fusion protein following MACO produced a 30% re-reduction in cortical stroke volume, whereas GDNF alone had no effect in stroke volume.¹⁶⁶ In addition, combination therapy with TfRMAB-GDNF and TfRMAB-TNFR following MCAO has an enhanced neuroprotection reducing the cortical stroke volume to 69%.¹⁶⁶ A brain penetrating form of BDNF may also represent another potential treatment for stroke (Table 1).⁴⁸ Studies in the Rhesus monkey with HIRMAB-BDNF suggested that it may be possible to reach therapeutic levels of this neurotrophic factor in brain following the systemic administration of the BBB-penetrating fusion protein.⁴⁸

3.3.8. Other BBB receptor-mediated transport systems. The Insulin-like Growth Factor-1 Receptor (IGF1R) is another member of the BBB receptor-mediated transport system family and has gained attention as potential shuttle for delivery of biologicals to the brain.^{13,167,168} Llama single-domain antibodies against the extracellular domain of the IGF1R were produced and engineered as bivalent antibody fused to either

human or mouse IgG-Fc, respectively. These constructs presented high affinity for the IGF1R, *i.e.*, low nM range, and a brain uptake of 12 nM 24 h after the IV administration of 15 mg per kg BW.^{167,168} *In vivo* efficacy was demonstrated with neurotensin fused to the transport IGF1R5-hFc vector, which induced dose-dependent decrease in core temperature. In addition, galanin chemically conjugated to IGF1R5-mFc reversed hyperalgesia.^{167,168} The IGF1R vector was also effective for brain delivery of IDS in rats, as another potential treatment for Hunter's MPSII syndrome.¹⁸⁸

Targeting BBB glucose transporter type 1 (GLUT1 or SLC2A1) and/or CD98hc, which represents the 4F2 heavy chain (SLC3A2) of the BBB large neutral amino acid transporter (LAT1 or SLC7A5), has been proposed to develop new brain shuttle systems.^{169,170} However, these are facilitated transporters or carrier mediated transporters (CMT), that they do not induce receptor-mediated transport across the BBB.^{8,9} Therefore, targeting these CMT with MAb and/or biologicals may induce endocytosis into the brain microvasculature, but there is no known mechanism for transport of CMT across the BBB. Thus, development of CMT as shuttle vectors for the brain should be the subject of another review article.

4. Long term treatment and safety of brain penetrating biotherapeutics

Extensive Good Laboratory Practice (GLP) pharmacological and toxicological studies were reported with HIRMAB fusion proteins in Rhesus monkeys.^{71,171,175} Comparable binding of HIRMAB-fusion proteins to human and Rhesus monkey organs in tissue-cross reactivity studies validated toxicological studies in primates.^{71,171} The solely adverse effect reported in primates with HIRMAB-IDUA (valanafusp- α) was hypoglycaemia at the high dose of 30 mg per kg BW.¹⁷² This was due to an allosteric agonistic effect of insulin, and it was completely preventable by performing the infusion of the drug in dextrose-saline.¹⁷² In paediatric Hurler MPS I phase I/II clinical trial with valanafusp- α , the test article was administered by weekly infusions of 1–6 mg kg⁻¹ in 5% dextrose-saline for 12 months.³² The fusion protein was well tolerated in >570 infusions, with an incidence of 5.9% of hypoglycaemic drug related adverse effect, which was transient and resolved within 10–20 min following snack or glucose sachet.³² Sixty-two % of the hypoglycaemic episodes were observed at the high dose of 6 mg kg⁻¹. Therefore, the hypoglycaemic incidence at the therapeutic dose of 1–3 mg per kg BW was just 2.8%.³² The mean blood glucose was normal throughout the 52 weeks trial, *i.e.*: 101 ± 20 mg dl⁻¹ over >3000 glycemia measurements.³² In the primate chronic toxicological study, no evidence of chronic toxicity was reported in any primate during the 6-month HIRMAB-IDUA toxicological study, nor in animals euthanized after the recovery period, and the pharmacokinetics was predictable over the entire dose range.⁷¹ No significant changes were found in physical exams, food intake, EKG, ophthalmoscopic exam, body weights, or organ weights in any of the treat-



ment groups relative to animals treated with the control article.⁷¹ Anti-drug antibodies (ADA) were seen in primates as response to the human fusion protein; but those were not neutralizing, as the end-of-study pharmacokinetics shows no change in plasma clearance from plasma or in plasma enzyme activity.^{71,173} In Hurler MPSI patients, there were just 10 Infusion Related Reactions (IRR), and this represents an incidence of just 1.7%. Of the latter, 60% of the IRR were observed in a single naïve patient in whom tolerance to drug developed by week 10th.³² It is postulated that the relative low rate in IRR may be due to the presence of Tregitopes in the constant region of human IgG, that may induce immunotolerance.¹⁷⁴

Chronic toxicological studies were also reported for the HIRMAb-IDS in the Rhesus monkey with weekly dosing to up to 30 mg per kg BW for 6 months.¹⁷¹ The weekly infusions were performed in dextrose-saline to prevent potential hypoglycaemic events.¹⁷¹ No adverse effect nor chronic toxicity were reported; therefore, the No Adverse Event Level (NOAEL) for the HIRMAb-IDS was set at 30 mg per kg BW.¹⁷¹ Pharmacology and safety studies were reported as well for the acute administration HIRMAb-GDNF in the Rhesus monkey.¹⁷⁵ This GLP toxicological study was aimed for an acute treatment of stroke with IV doses of up to 50 mg per kg BW fusion protein over a 60-hour period in 56 primates.¹⁷⁵ No adverse events nor neuropathologic changes were reported in the 2-week terminal toxicology study.¹⁷⁵ A study in a MPTP-PD model in the Rhesus monkey reported proliferative lesions in the pancreas of 4 of 7 animals at the low dose of 1 mg kg⁻¹ HIRMAb-GDNF, but not at the high dose of 5 mg kg⁻¹.¹⁶¹ The lesions reported at the low dose are observed in 30% of all human autopsies and they are not pre-malignant.¹⁷⁶ On the contrary, no pancreatic lesions were reported following 6 months of treatment with either HIRMAb-IDUA or -IDS fusion protein, respectively, with doses as high as 30 mg per kg BW per week.^{71,171} Chronic studies in mice with TfRMAb-GDNF fusion protein demonstrated no histological changes in brain and cerebellum, kidney, liver, spleen, heart, or pancreas; no change in body weight, and no change in 23 serum chemistry measurements.⁶⁶ A low-titre immune response directed against the variable region of the fusion protein was reported.⁶⁶ As shown for other HIRMAb-fusion proteins, these antibodies were not neutralizing.^{66,71,171}

An adverse effect of shuttle systems targeting the TfR is a decrease in circulating reticulocytes reported after acute dosing of a low affinity TfRMAb.¹⁷⁷ Mutation of the Fc effector function was reported to rescue the reduction in reticulocytes.¹⁷⁷ On the other hand, several chronic studies using high affinity TfRMAb did not report changes in circulating reticulocytes.^{51,54,66,135,135,147-150,157,158,163-165} The acute effect of TfRMAbs on reticulocytes appears to be transient and reversed by chronic treatments. Elimination of the effector function in the TfRMAb-EPO fusion protein was achieved by substituting Asn-Gly at residue 292. This mutation resulted in a markedly reduced C_{max} following either intravenous or subcutaneous administration.⁷³ The potential benefit of elimination of effector function may be offset by its rapid pharmaco-

kinetic clearance. Newer designs with TfR-Fab as transport domain – lacking the IgG-Fc region – may be preferred to minimize adverse effects associated with Fc effector function.³³ In the APP/PS1 model of Alzheimer's disease, chronic administration of the mouse TfRMAb-EPO fusion protein improved both hematology safety and behavioural/therapeutic outcomes compared with recombinant EPO alone.^{151,178} Chronic administration of hTfRMAb to Rhesus monkeys led to toxicity, including anaemia associated with suppressed blood reticulocytes, microglia activation and moderate axonal/myelin degeneration in the sciatic nerve.¹⁷⁹ Nevertheless, these haematologic and neuropathological effects may be attributable to Fc effector functions that were not specifically assessed in that study. On the contrary, a chronic study in the cynomolgus monkey conducted with pabinafusp alfa reported no effector function nor significant toxicity at doses up to 30 mg per kg per week for 26 weeks.⁶⁷ In the phase II-III clinical trial with pabinafusp alfa in Hunter MPSII patients, transient IRR were reported in 14 of 28 subjects. These events were clinically manageable and did not require discontinuation of the treatment. Serious adverse events in five patients were reported to be unrelated to the test drug, including one death due to respiratory failure and resultant hypoxic encephalopathy – both conditions associated with MPSII.³⁴ Trontinemab showed a good safety profile in a phase I/II clinical trial, with ARIA occurring in less than 5% of participants. These events were most likely attributable to the therapeutic Aβ MAb and not from the TfR-Fab transport domain (<https://www.alzforum.org/news/conference-coverage/trontinemab-fuels-hope-brain-shuttle-lift>).

Repeat IV dosing in animal models and/or human chronic studies represent complications of the study design. Thus, it would be preferred to dose test articles *via* IP or SQ. Studies in mice demonstrated that comparable brain levels of a brain penetrating IgG-fusion protein may be achieved with the administration of the test article IV, IP, or SQ.¹⁸⁰ The efficacy of TfRMAb-SGSH fusion protein was demonstrated in a mouse model of MPSIIIA mice with IP injections of 5 mg kg⁻¹ three times weekly for 6 weeks.⁵⁴ TfRMAb-EPO fusion protein was also effective in a mouse model of AD with IP or SQ injections of 3 mg kg⁻¹ BW.^{150,178} In addition, high plasma levels of HIRMAb were reported in the Rhesus monkey following chronic SQ administration, suggesting that protein therapeutics for the brain may be developed with chronic SQ administration on a weekly or twice weekly regimen.¹⁸¹

Both Tf and insulin receptors are also expressed in neuron and glial cells throughout the brain, as well as in peripheral organs, like liver.^{62,189,190} Therefore, potential adverse off target effects should be considered. However, in the case of lysosomal storage disorders, targeting both CNS and non-CNS organs resulted in beneficial therapeutic effects (sections 3.3.1 to 3.3.4). Moreover, no peripheral toxicity has been reported for other therapeutic payloads, *i.e.*: brain penetrating Aβ MAb (section 3.3.6.1).

The safety of the production process of IgG fusion proteins for GLP toxicological studies and for the GMP material used in clinical trials has also been reported.³⁷



5. Conclusions

Since the hypothesis that peptidomimetic biotherapies can be transported to the brain *via* BBB receptor-mediated transport systems was introduced in the 1980–90s,¹⁹¹ the efficacy targeting these BBB receptors was reduced to practice in numerous models of CNS disorders. The application of transport/shuttle systems targeting the HIR and TfR receptors at the BBB was discussed for chemical conjugated peptides, ASO and siRNA in section 3.1. The application of these RMT BBB systems to non-viral gene delivery, including brain tumours, was discussed in section 3.2. The application of brain penetrating IgG-fusion proteins to lysosomal storage disorders was discussed in sections 3.3.1 to 3.3.5. The use of brain penetrating IgG-fusion proteins in neurodegeneration, *i.e.*: Alzheimer's and Parkinson's disease, respectively, was discussed in section 3.3.6. Finally, the application of brain penetrating IgG-fusion proteins for stroke was discussed in section 3.3.7.

Even though multiple preclinical studies in primates projected therapeutic brain levels targeting the HIR in the pathological conditions listed in Table 1 (sections 3.3.1 to 3.3.7), most of the protocols currently being developed in academia and the biotech industry are focused on the TfR. GLP pharmacology studies in primates with HIRMAb fusion proteins showed no toxicity, and only minor acute hypoglycaemic episodes,^{71,171,175} also seen in the phase I/II clinical trial with valanafusp alfa in paediatric MPSI, which were resolved within 10–20 min following snack or glucose sachet.³² Potential safety concerns about targeting the TfR are related to reduction in reticulocytes due to a Fc effector related function.¹⁷⁷ Although, this adverse effect appears to be acute, and pabinafusp alfa shown no effector function nor significant toxicity in a toxicological study in the cynomolgus monkey for 26 weeks.⁶⁷ An advantage targeting the TfR in lieu of the HIR is that pre-clinical studies in mice and clinical trials in humans can be performed with the same human therapeutic construct, as studies in mice are performed in hTfR knockin mice.¹⁰⁵ Such studies could not be performed in HIR knockin mice because the abundance of the mouse BBB transferrin receptor is approximately 7-fold higher than that of the mouse BBB insulin receptor.^{182,183} Therefore, targeting the mouse BBB insulin receptor would produce lower levels of brain uptake. Targeting the BBB TfR in mice or either the HIR or the human TfR in primates resulted in a comparable brain uptake of 1–3% of the injected dose (sections 3.3.1 to 3.3.7). This relates to the abundance of these receptors at the BBB, which is comparable in humans.¹⁸²

The first generation of brain penetrating IgG-fusion proteins has been engineered with high affinity bivalent MAbs directed to HIR or mTfR (Tables 1 and 2, sections 3.3.1–3.3.7). These constructs produced brain uptake levels of 1–3% ID per brain in mice and primates,³⁷ which are comparable to the brain uptake of diazepam.⁶¹ The brain VD of these brain penetrating biotherapeutics were high and ranged from ~300 to 800 $\mu\text{L g}^{-1}$,³⁷ which are several-fold higher than the blood volume in brain, *i.e.*: 14–20 $\mu\text{L g}^{-1}$.^{38,62} The extent of BBB pene-

tration of these MAbs was quantified using the capillary depletion technique, which demonstrated transport across the BBB and no retention in the brain microvasculature.^{37,38,62} As discussed in section 2.1, hypotheses were postulated involving the affinity and valency of MAbs to produce shuttle systems with improved brain penetration.⁶³ Recent publications also aimed to fine-tune the affinity of TfRMAb for the BBB-TfR using the mouse specific 8D3 MAb.^{68–70} In summary, different tendencies were generated pursuing brain penetrating constructs with different characteristics, *i.e.*: (a) conventional design comprised on bivalent high affinity transport MAb, (b) high affinity monovalent transport vectors, and (c) low affinity monovalent transport shuttles. All 3 designs are currently in clinical trials. (a) Is represented by pabinafusp alfa, bivalent high affinity TfRMAb fused to IDS for MPSII.^{34,36} In the (b) design, a high affinity monovalent TfRMAb-Fab transport vector in lepunafusp alfa for MPSI and in postnafusp alfa for MPSIIIA, and a high affinity monovalent HIRMAb-Fab transport vector in GNR-055 for MPSII.^{33,39} (c) The low affinity monovalent design is found in trontinemab for Alzheimer's disease and in tvidenofusp alfa for Hunter's MPSII.^{35,53} High affinity transport MAbs have short plasma residence time, and low affinity MAbs present longer plasma residence time. The kinetics modelling indicated that the lower the affinity of the antibody for the TfR, the greater the ID required to maintain a given brain AUC.⁷² Although, trontinemab is administered at relatively low dose, *i.e.*: 3.6 mg per kg BW every 4 weeks.³⁵ A lower therapeutic dose may also be preferred to reduce potential adverse effects, as in the case of IgG-neurotrophic factor or -TNFR fusion proteins.^{46,73,140,141}

Most of the open clinical trials with brain penetrating biotherapies are focused on LSD (sections 3.3.1 to 3.3.5). The chance for successful trials is high, as the replacement of just 1–2% of normal enzyme activity in LSD is expected to be therapeutic.¹¹⁷ This is also supported by the fact that 5–20% of the population has ASA pseudo-deficiency, with 3–8% of normal levels of ASA enzyme activity in brain and no symptoms of MLD,¹¹⁸ and that just 0.5% of endogenous PPT1 activity is needed to reverse the neuropathology of CLN1.¹¹³ However, levels of the biotherapy in CSF may not be sufficient as primary efficacy endpoint, as all IgGs in plasma and are found in CSF *via* passage across a leaky choroid plexus, and that the ratio of any IgG in CSF/plasma is normally 0.1–0.2%.^{185,186}

Based on the data presented in this review, it is possible to reformulate virtually any protein-based therapeutic into a brain-penetrating IgG fusion protein therapeutic, provided that the therapeutic domain maintains its biological activity (Tables 1 and 2). In most constructs, the therapeutic domain is fused to the C-terminus of either the heavy or the light chain of the transport MAb domain (Fig. 6). The fusion to the heavy chain design places the therapeutic domain of the fusion protein in a dimeric configuration, which mimics the mature native structure of enzymes, neurotrophic factors, decoy receptors, and bispecific MAbs. Fusions to the light chain results in a flexible configuration, as in the case of HIRMAb-HEXA and HIRMAb-ASM (Fig. 6), which both form heterodimer com-



plexes with other proteins, *i.e.*, the GM2 activator protein and saposin C, respectively (section 3.3.5). It is also possible to engineer IgG fusion proteins where the therapeutic domain is fused to the N-terminus of the transport MAb. However, a glucuronidase fused to the N-terminus of HIRMAb showed a marked reduction in the affinity for the target receptor, to levels that would abolish its transport through the BBB.¹⁸⁴ On the contrary, in tividinofusp alpha, the IDS therapeutic domain is fused to the N-terminus of the EVT:IDS, which is spatially separated from the TfR binding site located near the C-terminus of the Fc region (Fig. 8). Regarding brain penetrating bispecific MAbs, different formats have been engineered (Fig. 10). The classical configuration with the therapeutic MAb in the form of scFv fused to the C-terminus of the heavy chain of the transport MAb, or in the reverse configuration, where the therapeutic MAb is in a full antibody form and the transport MAb is an scFv configuration fused to the C-terminus of the light chain of the therapeutic MAb (Fig. 10). The reverse configuration has the advantage of converting any MAb into a brain-penetrating tetravalent bispecific MAb. Finally, in trontinemab, the transport domain is a monomeric TfR-Fab fused to the C-terminus of the anti-A β gantenerumab (Fig. 10). Based on the data discussed, the evolution of the field resulted in a variety of designs enable engineering of brain penetrating biotherapeutics. Each design present advantages and disadvantages, for example: (i) full IgG constructs and/or constructs with IgG-Fc region may induce immune tolerance due the presence of Tregitopes,¹⁷⁴ it may facilitate brain efflux through the FcRn receptor;¹³³ but it may present adverse effects related to the Fc effector function; and (ii) Fab fusions without IgG Fc region may present improved brain residence time due to absence of FcRn binding, and no Fc effector function, but it may lack the benefits of Tregitopes.

In conclusion, a broad range of brain-penetrating IgG fusion proteins have been engineered, validated in various animal models of CNS disorders, and currently being evaluated in human clinical trials, with pabinafusp alfa being the first of this class of biologicals approved by a regulatory agency in Japan. Pending further drug development, other members of the brain-penetrating IgG fusion protein family discussed in the present review are positioned to become a new generation of pharmaceutical drugs for the treatment of human CNS disorders.

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication authorship.

Conflicts of interest

The author is the co-inventor of patents on the delivery of biological drugs to the brain.

Abbreviations

AD	Alzheimer's disease
ASA	Arylsulfatase A
ASM	Acid shingomyelinase
ASO	Antisense oligonucleotide
AUC	Area under the curve
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
DS	Dermatan sulphate
ECD	Extracellular domain
EGF	Epidermal growth factor
EGFR	EGF receptor
EPO	Erythropoietin
GAG	Glycosaminoglycans
GDNF	Glial-cell-derived neurotrophic factor
Gfap	Glial fibrillary acidic protein
GLB1	β -Galactosidase
GLP	Good laboratory practice
GLU1	Glucose transporter type 1
GUSB	Glucuronidase
HEXA	Hexoaminidase A
HIR	Human insulin receptor
HS	Heparan sulfate
ID	Injected dose
IDUA	Iduronidase
IDS	Iduronate-2-sulfatase
IGF1R	Insulin-like growth factor-1 receptor
IML	Immune liposome
IP	Intraperitoneal
IV	Intravenous
lacZ	β -Galactosidase gene
LAT1	Large neutral amino acid transporter type 1
LSD	Lysosomal storage disorders
MAB	Monoclonal antibody
MLD	Metachromatic leukodystrophy
M6P	Mannose-6-phosphate
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NAGLU	N-Acetyl-alpha-D-glucosaminidase
PD	Parkinson's disease
PNA	Peptide nucleic acid
PON1	Paroxonase-1
PPT1	Palmitoyl-protein thioesterase
PS	Permeability-surface area
RMT	Receptor mediated transcytosis
SA	Streptavidin
scFv	Single chain Fv
SGSH	Sulfamidase
SST	Somatostatin
SQ	Subcutaneous
TfR	Transferrin receptor
mTfR	Mouse TfR
rTfR	Rat TfR
TH	Tyrosine hydroxylase
TNF	Tumour necrosis alfa



TNFR TNF receptor
VD Volume of distribution

Data availability

No primary research results, software or code have been included and no new data were generated or analyzed as part of this review.

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