Molecular Therapy
Review

Targeting the central nervous system in lysosomal storage diseases: Strategies to deliver therapeutics across the blood-brain barrier

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Lysosomal storage diseases (LSDs) are multisystem inherited metabolic disorders caused by dysfunctional lysosomal activity, resulting in the accumulation of undegraded macromolecules in a variety of organs/tissues, including the central nervous system (CNS). Treatments include enzyme replacement therapy, stem/progenitor cell transplantation, and *in vivo* gene therapy. However, these treatments are not fully effective in treating the CNS as neither enzymes, stem cells, nor viral vectors efficiently cross the blood-brain barrier. Here, we review the latest advancements in improving delivery of different therapeutic agents to the CNS and comment upon outstanding questions in the field of neurological LSDs.

INTRODUCTION

Lysosomal storage diseases (LSDs) are a group of more than 70 inherited metabolic disorders characterized by deficient function of lysosomes, organelles whose function is to catabolize macromolecules. The lysosome contains an array of hydrolytic enzymes that, together with transporters, lysosomal membrane proteins, and targeting motifs are accountable for the proper functioning of the cell-recycling apparatus. Defects in any of these components result in the aberrant accumulation of undegraded macromolecules, or "storage products," disruption of cell homeostasis, cell dysfunction, and, in some cases, cell death.[1] Prevalence of each LSD is very low; however, when considered as a group they affect a significant minority of live births (12.1–25 per 100,000[2]). LSDs are genetically heterogeneous, and can be classified into subcategories depending upon the type of macromolecule involved (reviewed by Platt et al.[1]). These are multisystem diseases that affect different tissues and organs to a variable degree depending on lysosome/substrate distribution, and expression profile of the causative gene(s). Clinical symptoms range in severity depending upon the extent to which a specific LSD affects each cell type, tissue, or organ; however, 50%–70%[3,4] significantly affect the central nervous system (CNS), resulting in severe and progressive neurodegeneration. Brain damage commonly begins in early infancy but can also occur during adulthood in late onset forms. Neurological LSDs (summarized in Table 1) are often fast-progressing fatal diseases, therefore substantial effort has been made to develop effective treatments.

Currently, there are several experimental and clinical treatments available for specific LSDs with the collective aim of restoring enzyme function. Standards of care include (1) enzyme replacement therapy (ERT) to deliver exogenous enzyme directly to the patient[5]; (2) hematopoietic stem/progenitor cell (HSC) transplantation, in which patients receive either allogeneic or autologous HSCs, which are genetically modified *ex vivo* (HSC gene therapy) and are able to engraft the CNS, providing a source of functional enzyme[6,7]; and (3) substrate reduction therapy that utilizes small molecules to attenuate accumulation of specific macromolecules.[8] Ongoing clinical trials are evaluating improved standard of care approaches, especially for HSC gene therapy, while also testing alternative approaches. These include *in vivo* gene therapy, which delivers a healthy copy of the defective gene directly to patients' cells[9]; and chaperone therapy to guide correct protein folding of patients' aberrant enzymes to improve their catalytic function.[10]

Despite some of these treatment strategies being successful for specific forms of LSDs,[11] there are still a number of drawbacks. Each treatment has different limitations: ERT is immunogenic, must be administered regularly, and has limited efficacy in some organs[5]; HSC transplantation (HSCT) necessitates chemotherapeutic pre-conditioning and has a risk of transplant-associated morbidity and mortality[6,7]; substrate reduction therapy, like ERT, does not correct the primary defect and some molecules are associated with undesirable secondary side effects[8]; and a range of gene therapy vectors can be immunogenic.[12]

One limitation that is common to all these strategies is the inability, or limited ability, of therapeutic agents to cross the blood-brain barrier (BBB) and reach the CNS or, in the specific case of HSCT therapy, to engraft rapidly enough and in optimal numbers to prevent the rapid neurological deterioration that occurs in some LSDs. Consequently, in recent years there has been a strong focus on increasing delivery of therapeutic agents to the CNS. Innovations in CNS

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Table 1. Neurological LSDs
Summary of neurological LSDs, including details of defective gene, primary protein involved, and associated lysosomal storage product (adapted from Platt et al.[1]).

Neurological LSD (*gene*)	Primary defective protein (substrate/storage product)
Mucopolysaccharidoses (MPS)	
MPS I, also known as Hurler syndrome (*IDUA*)	α-L-iduronidase (dermatan sulfate, heparan sulfate)
MPS II (*IDS*)	iduronate 2-sulfatase (dermatan sulfate, heparan sulfate)
MPS III	
Type A (*SGSH*)	N-sulfoglucosamine sulfohydrolase (heparan sulfate)
Type B (*NAGLU*)	N-acetyl-α-glucosaminidase (heparan sulfate)
Type C (*HGSNAT*)	heparan-α-glucosaminide-N-acetyltransferase (heparan sulfate)
Type D (*GNS*)	N-acetylglucosamine-6-sulfatase (heparan sulfate)
MPS VII (*GUSB*)	β-glucuronidase (dermatan sulfate, heparan sulfate, chondroitin 6-sulfate)
Sphingolipidoses	
Fabry disease (*GLA*)	α-galactosidase A (globotriaosylceramide)
Gaucher disease type II, III, and perinatal lethal form (*GBA*)	β-glucocerebrosidase (glucocerebroside and glucosylsphingosine)
GM1 gangliosidosis types I-III (*GLB1*)	β-galactosidase (GM1 ganglioside, keratan sulfate, and oligosaccharides)
GM2 gangliosidosis	
Tay-Sachs (*HEXA*)	β-hexosaminidase (GM2 ganglioside, glycosphingolipids, and oligosaccharides)
Sandhoff (*HEXB*)	β-hexosaminidase (GM2 ganglioside, GA2 glycolipid, and oligosaccharides)
GM2 activator deficiency (*GM2A*)	GM2 ganglioside activator (GM2 ganglioside and glycosphingolipids)
Krabbe disease, also known as globoid cell leukodystrophy (*GALC*)	galactosylceramidase (galactocerebroside and psychosine)
Metachromatic leukodystrophy (*ARSA* and *PSAP*)	arylsulfatase A and prosaposin (sulfatides)
Niemann-Pick disease type A (*SMPD1*)	sphingomyelin phosphodiesterase (sphingomyelin)
Glycoproteinoses	
α-Mannosidosis types I, II, and III (*MAN2B1*)	lysosomal α-mannosidase (mannose-rich oligosaccharides)
β-Mannosidosis (*MANBA*)	β-mannosidase (Man(β1>4) N-acetylglucosamine)
Fucosidosis (*FUCA1*)	α-L-fucosidase (fucose-rich oligosaccharides, glycoproteins, and glycolipids)
Aspartylglucosaminuria (*AGA*)	aspartoglucosaminidase (aspartylglucosamine)
Schindler disease: types I–III (*NAGA*)	α-N-acetylgalactosaminidase (sialylated or asialo glycopeptides and glycosphingolipids)
Sialidosis type II (*NEU1*)	neuraminidase-1 (sialylated oligosaccharides and glycopeptides, LAMP1, and amyloid precursor protein)
Glycogen storage diseases (GSD)	
GSD II, also known as Pompe disease (GAA)	lysosomal α-glucosidase, also known as acid maltase (glycogen)
Lipid storage diseases	
Acid lipase deficiency, also known as Wolman disease (*LIPA*)	lysosomal acid lipase/cholesteryl ester hydrolase (cholesteryl esters, triglycerides, and other lipids)
Post-translational modification defects	
Mucolipidosis type II, also known as Inclusion-cell disease (*GNPTAB*)	N-acetylglucosamine-1-phosphotransferase subunits α/β (oligosaccharides, glycosaminoglycans, and glycosphingolipids)
Integral membrane protein disorders	
Danon disease (*LAMP2*)	lysosomal associated membrane protein 2 (cytoplasmic debris and glycogen)
Action myoclonus-renal failure syndrome (*SCARB2*)	lysosomal integral membrane protein 2 (unknown)
Sialic acid storage disease (*SLC17A5*)	sialin (sialic acids)
Niemann-Pick disease type C (*NPC1* and *NPC2*)	NPC intracellular cholesterol transporter 1 and 2 (cholesterol and sphingolipids)
Mucolipidosis type IV (*MCOLN1*)	mucolipin 1 (lipids and mucopolysaccharides)

(*Continued on next page*)

Table 1. *Continued*

Neurological LSD (*gene*)	Primary defective protein (substrate/storage product)
Neuronal ceroid lipofuscinoses (largely unknown heterogeneous mix of substrates)	
CLN1 (*PPT1*)	palmitoyl-protein thioesterase 1 (lipidated thioesters and saposins A and D)
CLN2 (*TPP1*)	tripeptidyl peptidase 1 (subunit c of mitochondrial ATP synthase)
CLN3 (*CLN3*)	battenin (subunit c of mitochondrial ATP synthase)
CLN4 (*DNAJC5*)	cysteine string protein (subunit c of mitochondrial ATP synthase)
CLN5 (*CLN5*)	ceroid-lipofuscinosis neuronal protein 5 (subunit c of mitochondrial ATP synthase)
CLN6 (*CLN6*)	transmembrane ER protein (subunit c of mitochondrial ATP synthase)
CLN7 (*MFSD8*)	major facilitator superfamily domain containing 8 (subunit c of mitochondrial ATP synthase)
CLN8 (*CLN8*)	ceroid-lipofuscinosis neuronal protein 8 (subunit c of mitochondrial ATP synthase)
CLN9 (gene unknown)	protein unknown (substrate unknown)
CLN10 (*CTSD*)	cathepsin D (saposins A and D)
CLN11 (*GRN*)	granulin (unknown)
CLN12 (*ATP13A2*)	cation-transporting ATPase 13A2 (inorganic cations)
CLN13 (*CTSF*)	cathepsin F (unknown)
CLN14 (*KCTD7*)	potassium channel tetramerization domain containing 7 (unknown)
Lysosome-related organelle disorders	
Chédiak-Higashi disease (*LYST*)	lysosomal trafficking regulator (size and movement of lysosomes)

delivery have been recently discussed from the perspective of nanoparticles[13] and small molecules.[14] However, methods to improve delivery of enzymes, stem cells, and viral vectors to the CNS have not been reviewed in recent years. This review focuses upon methods to increase delivery across the BBB, with emphasis on the latest advancements in targeting HSCT, ERT, and viral vectors to the CNS, and discusses the future of CNS-directed LSD therapy.

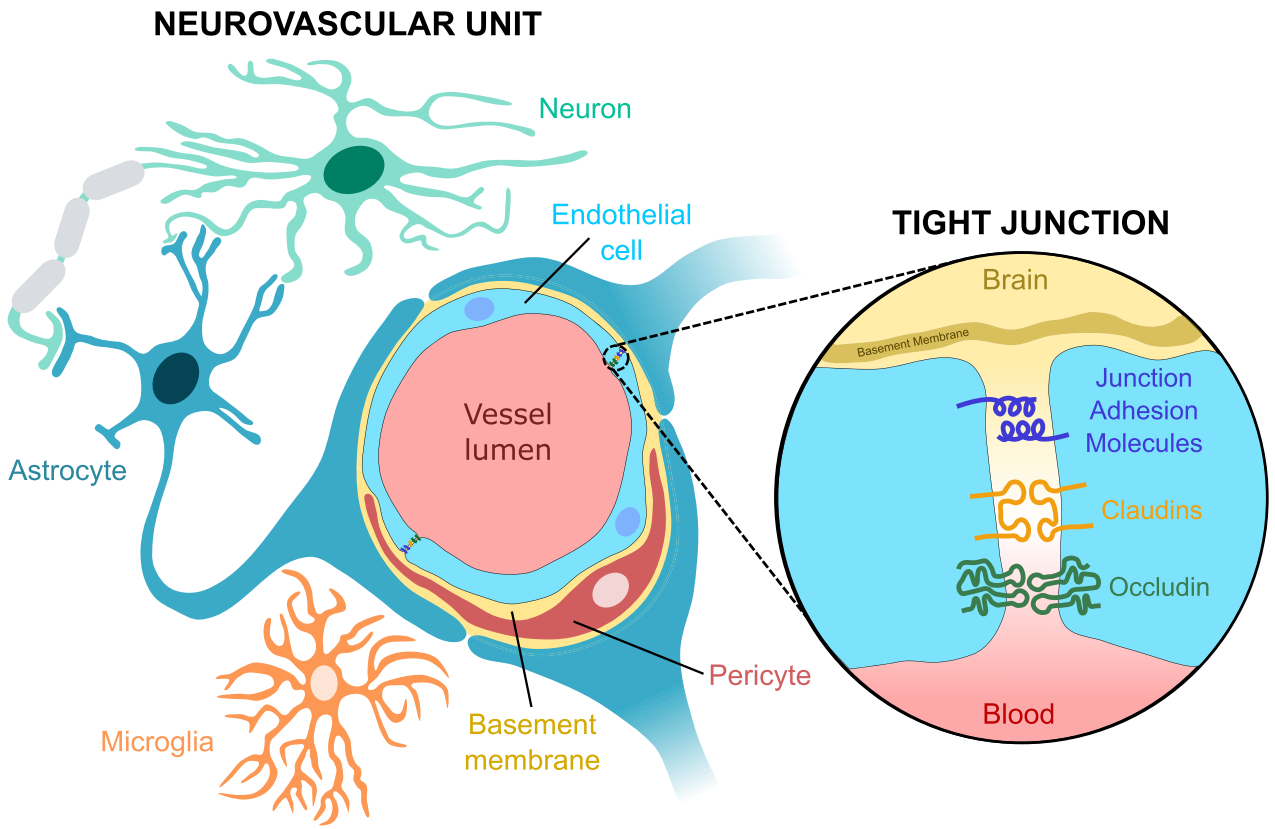
THE BLOOD-BRAIN BARRIER (BBB)

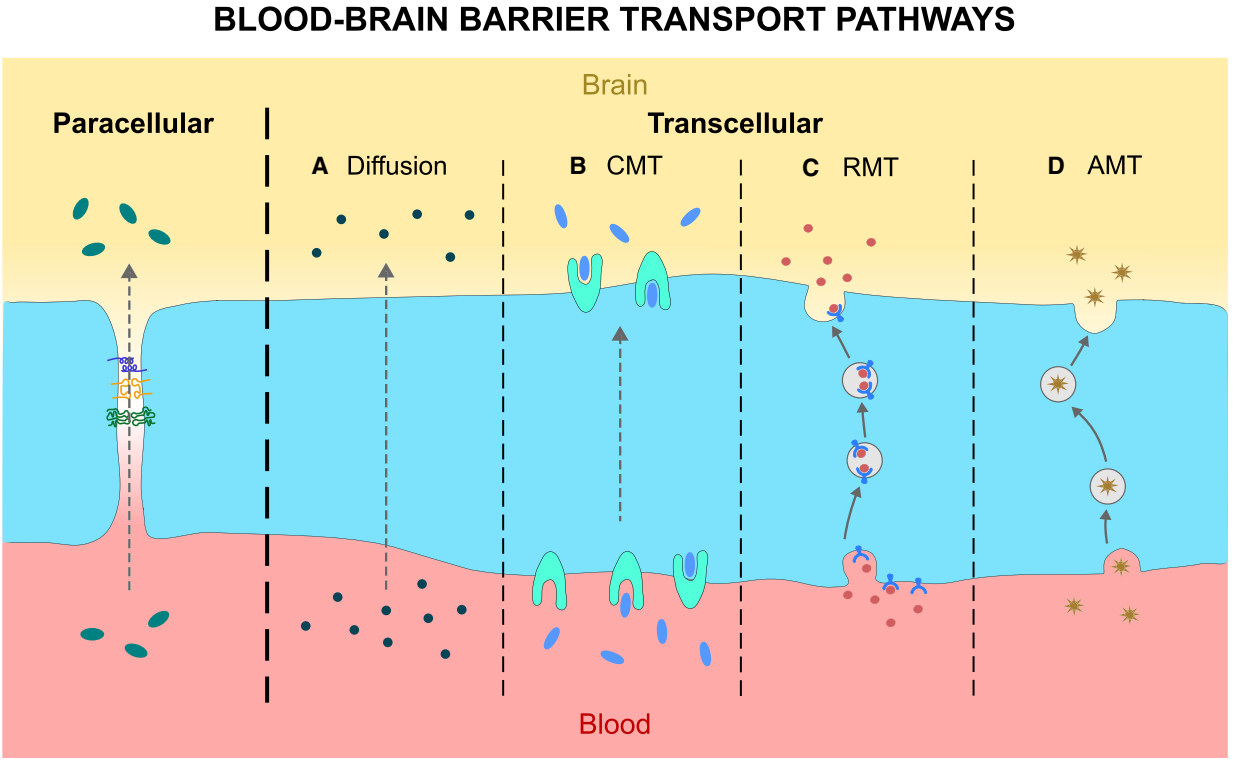
The BBB is a selectively permeable barrier between the CNS and the systemic circulation that controls exchange of solutes and protects the brain from toxins and potential pathogens circulating in the bloodstream.[15] It is comprised of neurovascular units, in which brain cells closely interact with the vasculature. The neurovascular unit involves multiple cell types: endothelial cells, pericytes, astrocytes, neurons, and microglia. Endothelial cells are the primary component and are supported by pericytes, perivascular cells that embrace the vessels and provide them with stability. Astrocytic endfeet ensheath almost the entire abluminal surface of microvessels,[16] and neurons and perivascular microglia interact with these cells to establish the neurovascular unit (Figure 1). Brain endothelial cells are especially vital for restricting BBB permeability, and have particular properties that enable them to perform this function, including (1) reduced transcellular flux, (2) lack of fenestrations, (3) greater mitochondrial density to assist rapid metabolism, (4) specialized transport systems, and (5) high electrical resistance as a result of an increased number of tight junctions between endothelial cells compared with other tissues and organs[17] (Figure 1). Multiple proteins are involved in tight junctions, namely junctional adhesion molecules, claudins, zonular occludens, and occludin.[15] Under normal conditions, they prevent molecules from leaking across the BBB through the paracellular transport pathway, which represents one of the two main transport routes across the BBB (Figure 1). Alternatively, molecules can move transcellularly with some crossing the BBB by passive diffusion, while most require assistance from carrier proteins (carrier-mediated transcytosis), receptors (receptor-mediated transcytosis), or vesicles (adsorptive-mediated transcytosis).[17]

However, in pathological conditions BBB integrity can be disrupted, allowing passage of substances that would normally not be able to cross. In the case of some CNS diseases (including Alzheimer's and Parkinson's), systemic diseases (e.g., diabetes mellitus and chronic cerebrovascular disease), and viral infections (e.g., viral encephalitis), disruptive remodeling of tight junctions results in reduced BBB integrity, leading to neuroinflammation, which further contributes to increased BBB permeability.[18–23] A greater understanding of the role of neurovascular units and tight junctions in the transport of therapeutic agents across the BBB, and being able to manipulate transport to increase delivery, may be vital for the effective delivery of therapeutic agents to treat the neurological component of LSDs. In the following sections, we explore how current treatments have been modified to improve stem cell, enzyme, and viral vector delivery across the BBB, including methods that exploit aspects of BBB transport pathways or bypass the barrier altogether.

BLOOD-BRAIN BARRIER (BBB) MANIPULATION

Several methods have been employed to disrupt the BBB with the aim of temporarily increasing permeability for LSD therapeutic agents (reviewed by Hersh et al.[24]).





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Here, we briefly explore both non-selective and selective methods that assist delivery of specific cells and/or enzymes.

Focused ultrasound

The use of magnetic resonance thermometry to guide focused ultrasound pulses in the presence of microbubbles is able to briefly compromise BBB permeability. Ultrasound pulses cause the microbubbles to expand and contract, temporarily separating endothelial tight junctions, which facilitates passage of therapeutics without allowing pathological events to occur.[25] In relation to neurological LSDs, the method has been used to deliver GFP-labeled neural stem cells to wild-type rat brains,[26] and to transport enzyme across the BBB in an MPS I murine model, restoring 75% of normal enzyme activity in the treated brain hemisphere.[27] Investigation of this method's safety and feasibility is underway in a number of neurological diseases and is employed in a phase I trial delivering Cerezyme (an analog of the β-glucocerebrosidase enzyme, which is also defective in the LSD Gaucher disease) across the BBB in Parkinson's disease patients (ClinicalTrials.gov NCT04370665).

Hyperosmotic agents

Intravenously delivered hyperosmotic agents increase BBB permeability by shrinkage of brain endothelial cells and consequent tight junction widening.[28] This temporarily augmented permeability allows a generalized increase in migration of substances from the bloodstream. The hyperosmotic agent mannitol has been used in murine models to deliver adeno-associated viral (AAV) vectors to the CNS in Sandhoff disease,[29] MPS IIIB,[30,31] and CLN2 deficiency,[32] showing enhanced delivery and greater therapeutic effect. However, the potential for toxic substances to cross the BBB during the period of non-selectively enhanced permeability, or for cerebral edema to occur if mannitol enters the brain, has limited its use in patients despite its clinical safety profile.[33]

Receptor stimulation

Receptor stimulation can increase delivery of enzymes across the BBB by relocalizing receptors to the luminal surface of brain endothelial cells. Studies in the LSD field have predominantly focused on the mannose 6-phosphate (M6P) receptor, a transport mechanism in the brain present during early post-natal development but lost during maturation.[34] Murine studies have shown that administration of epinephrine[35] or retinoic acid[34] stimulates M6P receptors and significantly elevates M6P-mediated transport of the lysosomal enzyme β-glucuronidase (P-GUS, defective in MPS VII) across the BBB. Further work involving direct stimulation of specific adrenoreceptors with α1/2 agonists suggested that increased enzyme uptake was likely due to redistribution of M6P receptors from an intracellular pool to the intra-luminal surface of brain microvascular endothelial cells.[34,36] These studies suggest that manipulation of receptor-mediated transport is a viable method for increasing selective delivery of enzymes across the BBB.

ENZYME REPLACEMENT THERAPY (ERT)

The concept of ERT as a potential treatment for LSDs (reviewed in Solomon and Muro[5]) first arose in the mid-1960s; however, a further three decades of development were required to generate the first effective, clinically approved ERT. ERT entails administration of fully functional exogenous enzyme to the patient, mainly via intravenous (i.v.) injection. The enzyme is taken up by patients' cells via endocytosis and trafficked to the lysosomes, where it compensates for endogenous enzyme dysfunction. ERT's limitations have been extensively reviewed elsewhere[5]; the major one of relevance to neurological LSDs is the inability to treat organs that are difficult to access—particularly the musculoskeletal, cardiovascular, ocular, and central nervous systems.[1] In the following sections, we review strategies employed to circumvent this limitation.

Enzyme modification

Fusion proteins

Modification of the therapeutic enzyme with an exogenous protein subunit might enable interaction with a specific receptor to increase CNS uptake. Multiple fusion proteins have been tested for efficacy in augmenting CNS delivery in LSD murine and/or primate models, including an acidic amino acid tag,[37] the fat-binding apolipoprotein E,[38–40] and importantly antibody conjugates targeting endogenous BBB transport receptors, including the insulin receptor[41–43] and the transferrin receptor.[44] Antibody-conjugated enzymes harness the receptor-mediated transport pathway to cross the BBB into the CNS. Results from *in vivo* studies demonstrated reduction of substrates and neuroinflammation in MPS II murine and primate models,[43,44] and highlighted a pharmacological safety profile.[41,42] A number of clinical trials (NCT03128593, NCT03568175, NCT04573023) have pursued this further; following a successful phase I/II trial of iduronate-2-sulfatase fused with an anti-human transferrin receptor antibody in MPS II patients,[45] results of a phase II/III study showed significantly reduced substrate accumulation in both the CNS and peripheral tissue, in addition to positive neurocognitive changes, while demonstrating a clinical safety profile consistent with current standards of care.[46] This strategy has now been approved for clinical use in Japan.[47]

Chemical modification

An alternative to fusion proteins is the chemical modification of lysosomal enzymes to alter receptors' affinity, allowing an elevated blood concentration of the therapeutic enzyme to maintain a high concentration at the BBB for prolonged periods. This approach has been tested in MPS VII[48,49] and MPS IIIA[50–52] murine models, showing significant reduction in CNS lysosomal storage biomarkers.[48–52]

Figure 1. The neurovascular unit and blood-brain barrier transport pathways

Graphical depiction of the neurovascular unit (NVU), the fundamental anatomical and functional unit of the blood-brain barrier (BBB), including the key protein components of tight junctions (TJs) between brain endothelial cells which control the paracellular transport pathway. Alternatively, molecules may be transported transcellularly via passive diffusion (A), carrier-mediated transcytosis (CMT) (B), receptor-mediated transcytosis (RMT) (C), or adsorptive-mediated transcytosis (AMT) (D).

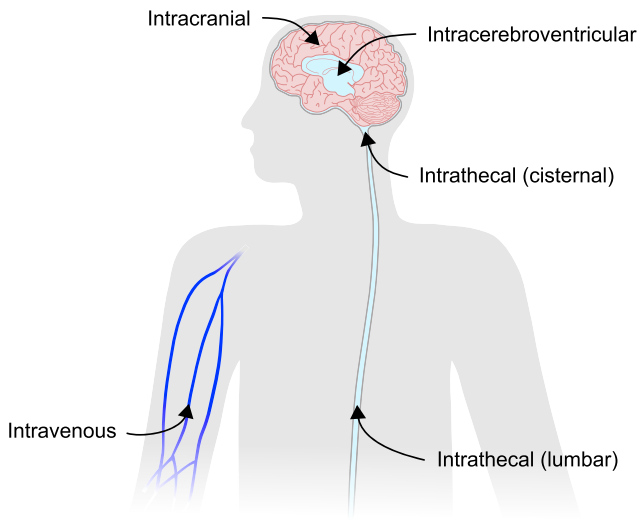


Figure 2. Clinically relevant delivery routes for LSD therapeutic agents
Graphical summary of the injection routes used to deliver enzyme replacement therapy (ERT), stem cells, and viral vectors in neurological LSDs.

Delivery

Injection routes

A range of different injection routes have been tested for ERT to improve enzyme delivery (Figure 2). Beyond traditional i.v. injection, intracerebroventricular (i.c.v.) injection[53,54] has been reported most extensively in recent literature in comparison with intrathecal (i.t., lumbar and cisternal),[53] or i.v. injection,[55] or to control conditions.[54,56–63] A number of studies have reported that i.c.v. is effective for ERT in multiple neurological LSD animal models.[53–55,57–62] However, these studies raise an important issue as sufficient enzyme delivery for therapeutic effect[59] remains a challenge. Treleaven et al. observed that less than 1% of the total ERT dose reached the CNS of a Niemann-Pick type A (NPA) murine model. Higher doses did not increase this percentage, suggesting that the enzyme uptake mechanism is saturated. However, while this is an extremely small proportion, it was distributed widely throughout the CNS, and previous work in the same murine model[61] demonstrated significant reduction of storage product levels and partial alleviation of motor abnormalities. The study proposed ERT scaling by CNS weight to maintain this therapeutic level in larger rodents.[59] Work in the NPA model raised a second potential limitation with i.c.v. delivery; despite therapeutic effect on the CNS as a whole, they observed a steep gradient in therapeutic enzyme from outer to inner brain regions, raising the possibility that therapeutic correction may be less successful in deeper tissue.[60] However, elevating the concentration of therapeutic enzyme may trigger an immune response against the exogenous enzyme, as was observed in a few MPS I mice given high-dose i.v. ERT.[64] Thorough investigation of toxic effects of high-dose ERT, and the impact upon CNS therapeutic correction, are required.

Other CNS-targeted ERT injection routes, including i.t., intranasal (i.n.), and intracisternal (i.c.), have been tested to varying degrees. i.t. and i.c. methods have been trialled in a similar range of animal models to i.c.v.,[56,65–76] with i.t. being shown to have greater benefit over i.v. in a single MPS IA patient.[65] i.n. delivery has only been tested in a murine model of MPS I,[77,78] and, similar to i.c.v. injection, only a very minimal percentage of the total dose of therapeutic enzyme (estimated 0.001%) reached the brain.[78] Despite studies reporting a predominantly positive effect on the neurological pathology, these injection routes entail reduced quantity of administered enzyme and consequently a reduced effect in deep brain tissues.[56,66,68,70,73,77,78]

Few studies have directly compared the effect of different ERT injection routes on LSD CNS pathology. i.c.v. proved more therapeutically effective than i.c. injection in two studies[53,55] despite being the most invasive.[53] Comparison between i.c.v. and i.t. has shown mixed results; in a canine model of MPS II, i.c.v. injection of ERT was superior, with correction of deep brain tissues[55]; however, in wild-type non-human primates and dogs i.t. delivery gave better results,[67] which was supported by a small-scale trial of i.t. injection in MPS II mice by the same group; however, no mice were injected using the i.c.v. route, limiting direct comparison in the disease model setting.[67] Altogether, these studies suggest that i.c.v. injection is the most effective for delivering ERT to the CNS in LSD models; however, the concerns regarding non-homogenous delivery throughout the brain and the limited percentage of treatment delivered to the CNS (which, albeit small, is sufficient to exert a therapeutic effect) suggest that other strategies may need to be employed.

Delivery vehicles

Delivery vehicles, such as nanoparticles,[13] extracellular vesicles,[79] polymersomes,[80,81] and quantum dots,[82] have been explored to improve enzyme delivery to the CNS in LSDs. While quantum dots have only been investigated in an *in vitro* setting,[82] successful *in vivo* studies have been conducted with polymersomes,[81] extracellular vesicles,[79] and nanoparticles; of these, nanoparticles have been researched most extensively. Multiple studies have employed nanoparticles to successfully deliver therapeutic enzymes to the CNS of Gaucher disease,[83] Krabbe disease,[84] and MPS II murine models, reporting reduction of storage products to non-pathological levels[85] (for a thorough review of the role of nanoparticles in LSD treatment up to 2016, please refer to Martín-Banderas et al.[13]). However, it is important to note that not all CNS LSDs are amenable to treatment using nanoparticles; three different nanoparticle formulations tested in an MLD murine model showed no increase in CNS enzyme levels, perhaps due to the therapeutic enzyme itself interfering with the targeting of the nanoparticles to the CNS.[86] The authors speculate that this could be due to interference of the enzyme's charge or side-chain oligosaccharides with surfactant coating or apolipoprotein recruitment, which are reported to be key mechanisms in BBB transport of nanoparticles. Therefore, it is possible that other delivery vehicles may also be limited by this issue.

Delivery devices

Initially, subcutaneous delivery devices were designed to enable continuous delivery of therapeutic enzymes to the CNS.[87] Devices

that deliver therapeutic enzymes via the i.c.v.[88–91] or i.t. route[92,93] were tested in the past decade and proved effective in MPS,[88,89,93] MLD,[90] and NCL[91] murine models. In 2017, an infusion pump that delivers into the cerebrospinal fluid was tested in a canine model,[87] but the study concluded that repeated i.c. or intra-spinal delivery was more effective. Furthermore, continuous delivery of therapeutics via these devices necessitates storage of the enzyme at body temperature for prolonged periods, which is likely to compromise enzyme stability and therefore limit utility. Consequently, research focus has now shifted toward devices with no indwelling enzyme reservoir. An i.t. drug delivery device utilized for monthly dosing was tested in a clinical trial for MPS II patients[92] (ClinicalTrials.gov Identifier NCT02055118); early results indicated a promising 80% reduction in storage substrate; however, over 50% of the trial participants had their device removed because of significant adverse events,[92] either due to the device breaking or the infusion cannula migrating away from the delivery site. Recent trials of a new i.c.v. device (Ommaya reservoir) in MPS IIIB patients have proven more effective (EudraCT 2017-003083-13; ClinicalTrials.gov NCT02754076 and NCT03784287), and the device has been applied to delivery of an ERT approved for i.c.v. dosing in MPS II patients.[94]

Convection enhanced delivery

One alternative strategy that has predominantly been applied to augment i.c.v. delivery for brain tumor treatment is convection-enhanced delivery,[95] where catheters are stereotactically inserted and, using image guidance, directed into the interstitial spaces before an infusion pump is used to drive delivery, therefore not requiring a high concentration of the therapeutic agent.[95] The only *in vivo* application of this strategy for Gaucher disease[96] ERT showed progressive and complete filling of the CNS target regions with therapeutic enzyme, while a trial in a single patient with type 2 Gaucher disease demonstrated safety.[96] Another clinical study evaluated safety of convection-enhanced delivery for gene therapy agents in late infantile NCL patients, reporting no adverse effects of the procedure and enzyme infusion rates between 50% and 90%.[97] However, there has been very limited further testing of this method in neurological LSDs, perhaps due to the range of risks associated with this procedure, primarily backflow, air bubbles, and flow within brain tissue.[95]

STEM AND PROGENITOR CELL TRANSPLANTATION

The requirement for a permanent, long-term fix that delivers lysosomal enzyme to all affected tissues in LSD patients has pushed scientists to look at other treatments beyond ERT. One promising alternative is stem and progenitor cell transplantation, which can generate lifelong tissue-resident sources of functional lysosomal enzyme that can relieve both somatic and neurological pathology.[6] Stem and progenitor cells are injected into the patient, where they engraft in affected tissues, contribute to the patients' resident cell populations, and secrete functional enzyme. The ability of stem and progenitor cells to potentially cross the BBB and provide cross-correction in the CNS has led to this strategy being trialled for a range of neurological LSDs. To date, hematopoietic stem and progenitor cells (HSCs) have been most commonly trialed in LSD animal models, and also human patients.[6,7] Other stem cells have been used for transplantation specifically targeting the CNS in LSDs, including neural stem cells and, to a lesser extent, mesenchymal stem cells.

Analysis of neural and mesenchymal stem cell transplants for CNS LSDs can be found in a number of recent reviews[98–100]; herein, we focus on HSC transplantation (HSCT) as the most promising strategy. HSCs can either be isolated from a healthy donor (allogeneic transplantation) or in an autologous manner using the patients' own genetically modified cells to provide a healthy copy of the mutated/non-functional gene.[6]

A yet-to-be-identified subpopulation of transplanted HSCs is able to cross the BBB following the use of specific chemotherapy or irradiation-based conditioning regimes and replace tissue resident microglia in the CNS.[101,102] The newly generated microglia secrete functional lysosomal enzyme, which can be taken up by neighboring enzyme-deficient brain cells in a process called cross-correction (Figure 3, "cross-correction in the brain" panel).[6,7,98,99,103] At the same time, differentiation of HSCs (which do not engraft the CNS) reconstitutes the entire hematopoietic system, thereby providing a peripheral source of therapeutic enzyme (Figure 3, "reconstituting hematopoietic lineages" panel). However, treatment of the CNS remains a challenge. In HSCT, cellular engraftment is not instantaneous, and gradual expansion of the transplanted cell population is required before lysosomal enzyme activity can be restored.[6,99] During this period, neurological symptoms often progress, which significantly reduces the impact of HSCT.[6] Furthermore, efficacy of HSCT in the CNS can be limited by (1) insufficient quantity of transplanted cells being trafficked to the CNS or (2) not enough functional lysosomal enzyme from engrafted cells being expressed in the CNS.[99] At present, most studies in this field are designed to improve the ability of stem cells to secrete functional enzyme once they have engrafted the CNS, rather than increasing the absolute number which cross the BBB, because this aim is more achievable with current knowledge and technologies. In the coming sections, we explore innovative strategies targeted to the CNS pathology of LSDs.

Pre-conditioning agent

In bone marrow transplants, a pre-transplantation chemotherapeutic conditioning regime is essential to deplete patients' resident HSCs and, possibly, resident microglia,[102,104] which facilitates engraftment of an HSC subpopulation in the CNS following transplantation[102] (Figure 3). The most widely used pre-conditioning agent, busulfan, has been demonstrated to deplete resident microglia more effectively than alternative conditioning regimes (irradiation or treosulfan) in mice,[102] specifically by causing microglial senescence and exhaustion of their regenerative ability.[105] Some studies suggest that busulfan could also be responsible for vascular injury and BBB disruption,[106,107] hypothesizing that a perturbed BBB could be accountable for the increased HSC engraftment. However, recent work by Cartier and colleagues suggests a non-inflammation- or non-BBB-disruption-induced permissive engraftment following busulfan conditioning.[105] Busulfan is associated with significant systemic toxicity

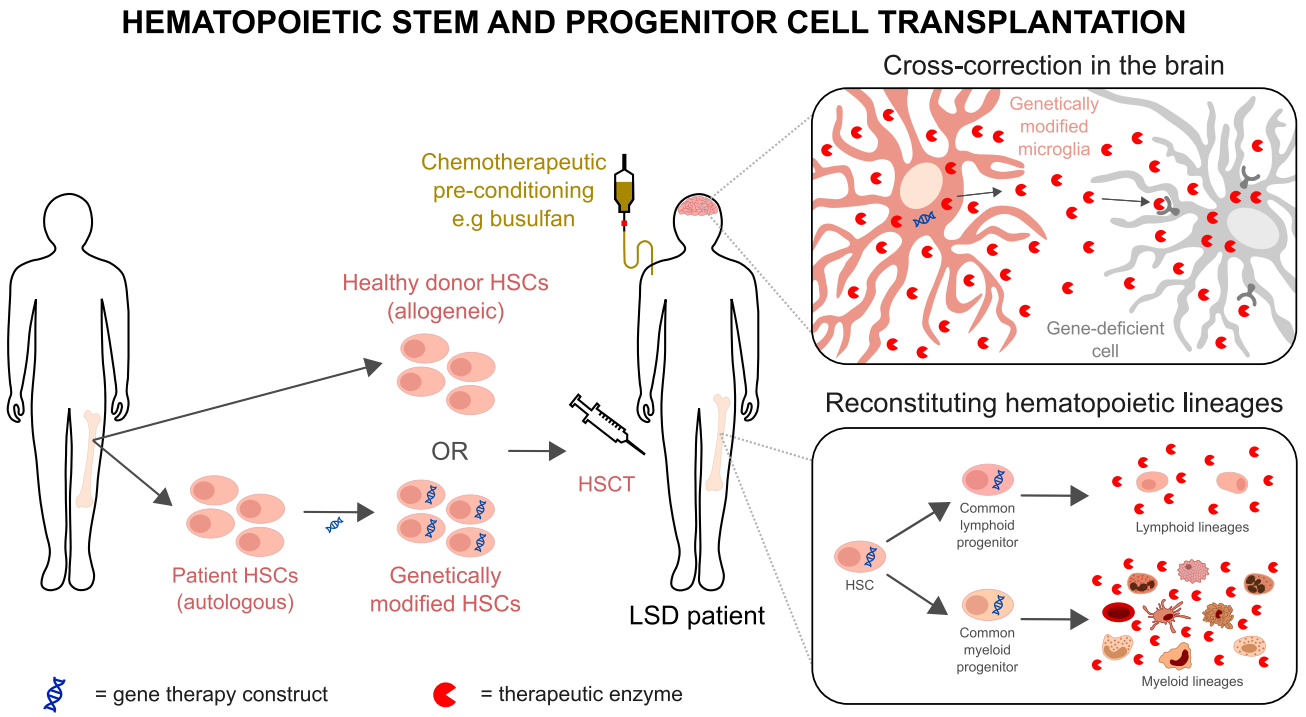


Figure 3. Overview of HSCT approaches

Graphical summary of HSCT using either allogeneic donor cells, or genetically modified patient cells to secrete a supraphysiological level of the defective enzyme. HSCT mediates therapeutic effect in the central nervous system by an HSC subpopulation crossing the BBB, engrafting the CNS, and generating genetically modified microglia, which provides a source of therapeutic enzyme to cross-correct neighboring enzyme-deficient brain cells.

in patients[108]; in addition, in mice it has been shown to cause a permanent inhibition of adult neurogenesis, suggesting a potential cognitive deficit for patients undergoing this regime[105] and emphasizing need for the future development of alternative pre-conditioning strategies with lower toxicity.

In this direction, antibody-based pre-conditioning regimes with reduced toxicity have been tested in mice,[109–111] and regimes that specifically target the hematopoietic lineages have successfully been used in severe combined immunodeficiency patients,[112] or immunocompetent mice and dogs.[113–115] However, the ability of antibody-based regimes to deplete resident microglia in the CNS and allow neurological engraftment of transplanted HSCs has not been determined.

Another option might be brain-targeted conditioning; this could potentially improve treatment efficacy in the CNS of neurological LSD patients. A newly synthetized and highly selective brain penetrant CSF1R inhibitor, PLX5622, has been used for extensive and specific microglial elimination in a murine model of Alzheimer's disease.[116] Moreover, in a recent study wild-type mice were pre-treated with PLX5622, lethally irradiated, and then received a bone marrow transplant. Mice receiving the CSF1R inhibitor showed a depletion of microglia and subsequent microglia replacement at the CNS-wide scale (around 90%) compared with non-treated mice, which show a minimal engraftment only in specific regions.[117]

Ex vivo stem cell gene therapy enhancement

When considering the two sources of therapeutic enzyme generated by HSCT, namely the peripheral cells of the reconstituted hematopoietic system and the tissue-resident macrophages, *ex vivo* gene therapy of autologous HSCs can be utilized in two ways to deliver a greater level of therapeutic benefit to the CNS. Firstly, by engineering vectors so that each genetically corrected cell secretes a supraphysiological level of enzyme, therapeutic benefit might be achieved in the CNS even with a limited number of engrafted cells. Moreover, studies have demonstrated that even a modest increase in enzyme activity in the CNS can provide therapeutic benefit; for example, restoring enzyme expression to 3.7% of wild-type levels in MPS II mice following HSCT was sufficient to correct CNS disease phenotype.[118] Secondly, modifying the enzyme sequence in the gene therapy construct so that therapeutic enzyme produced by peripheral hematopoietic cells can cross the BBB more easily also potentially enhances therapeutic effect in the CNS. Many of the methods of HSCT gene construct modification overlap with previously discussed enzyme modifications. In addition, HSC gene therapy for LSDs has been reviewed in depth by Biffi,[6] therefore we only briefly discuss it here.

Enzyme modification has been achieved in the HSC gene therapy setting by improving, before HSC transduction, the characteristics of the viral vectors used, or the therapeutic genes they contain. Similar to in ERT, fusion proteins have been included in the gene therapy

constructs to increase uptake by the CNS.[38,118] Other modifications of the therapeutic construct have focused on careful choice of promoters of gene expression. Appropriate choice and manipulation of the promoter could achieve production and secretion of supraphysiological levels of functional enzyme, and potentially increase its uptake by enzyme-deficient brain cells.[119,120] For example, utilization of the myeloid promoter CD11b to promote expression of the codon-optimized therapeutic enzyme specifically in myeloid cells (including microglia) and not in progenitors or other hematopoietic cells (to avoid potential toxicity) has proven beneficial in the CNS of MPS IIIA[121] and MPS IIIB[122] murine models, and has been taken further for MPS IIIA treatment with completed pre-clinical safety studies.[123]

An alternative approach to construct modification, which has been applied to a gene therapy construct but not yet combined with HSC gene therapy, focuses on promoting enzyme secretion and increasing post-translational activation speed in MPS IIIA[124,125] and MPS VII[126] mice. Further investigation is required to ascertain whether these concepts could perhaps be applied to HSC gene therapy for neurological LSDs. Both modification of the enzyme, or promoting its expression via editing of the gene therapy construct, have resulted in improved pathology correction in neurological murine LSD models, and represent a valid approach to targeting the CNS in LSDs. However, neither of these methods assist infiltration of the CNS by stem and progenitor cells.

Overall HSC gene therapy has shown to be effective in targeting the neurological pathology in LSDs[127–132] (and reviewed in Biffi[6]). HSC gene therapy clinical trials in MLD[127–129] and adrenoleukodystrophy (ALD)[130–132] patients showed high levels of therapeutic enzyme expression, reduction of storage products, and improvement of the clinical phenotype. Based on the efficacy and safety profile, the European Commission granted approval for marketing of HSC gene therapies for MLD and ALD at the end of 2020 and 2021, respectively.[133,134]

Administration routes

There has been less extensive investigation of transplantation administration routes in HSCT than in ERT for neurological LSDs; however, similar injection sites have been tested for stem and progenitor cell delivery to the CNS (Figure 2). Studies in MPS VII[135] and MPS I[136] murine models support the use of i.c.v. delivery to increase therapeutic effect in the CNS. Work by Capotondo et al. provided fundamental insight into the success of engraftment and fate of transplanted cells, demonstrating that HSCs do engraft the CNS, and give rise to microglia-like cells with biochemical characteristics matching bona fide microglia.[101] Comparison with conventional i.v. delivery provided evidence for i.c.v. injection leading to more rapid engraftment of the CNS and a greater abundance of therapeutic enzyme in a murine model of MLD.[102] Combined, these studies support i.c.v. delivery to improve therapeutic benefit in the CNS of LSD patients.

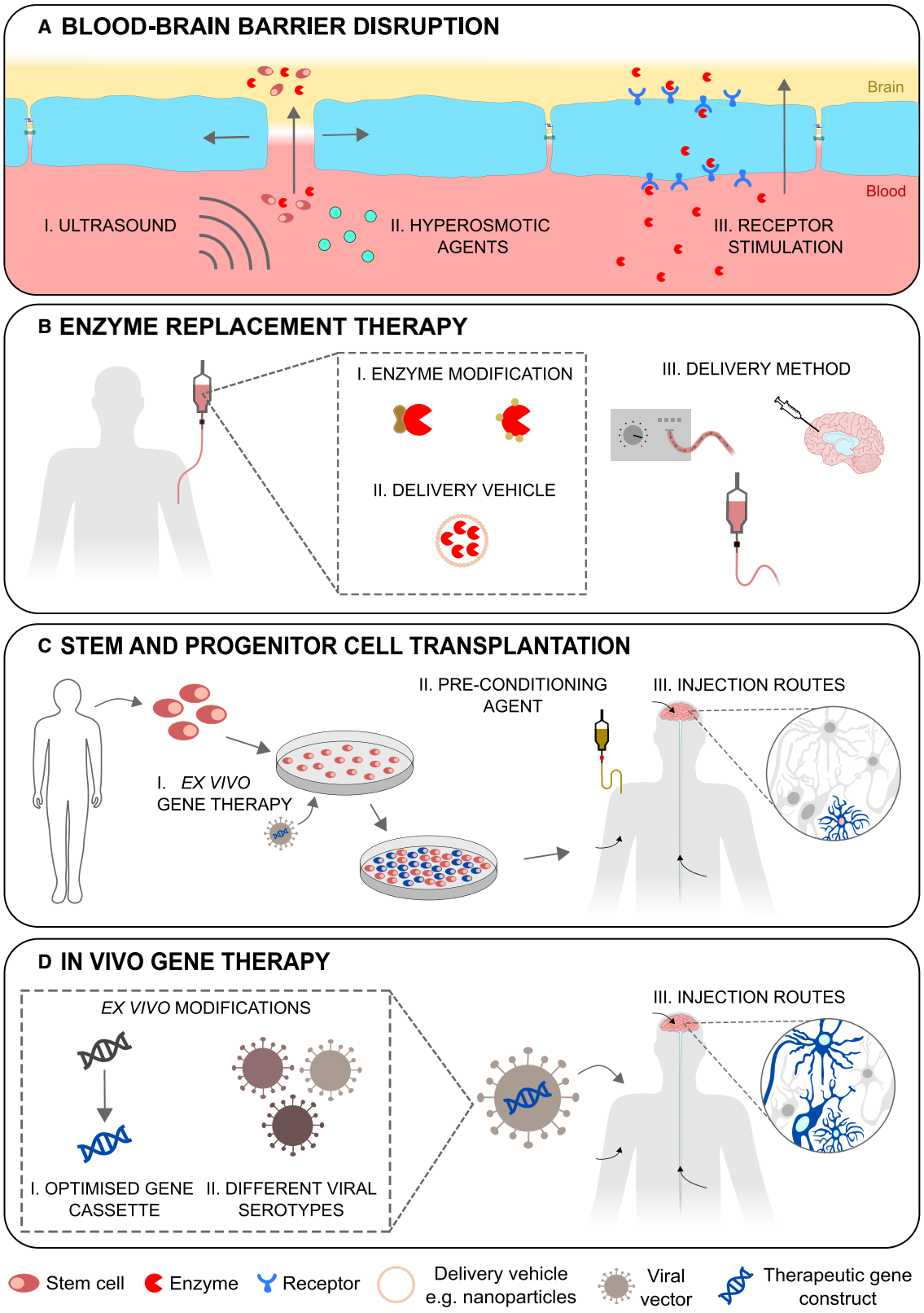
IN VIVO GENE THERAPY

Whilst we have already discussed using viral vectors for *ex vivo* gene therapy, we have not yet considered them as an independent treatment option. *In vivo* gene therapy involves delivering the therapeutic gene directly to patients' cells using a viral vector. In LSDs, gene therapy facilitates expression of therapeutic concentrations of functional lysosomal enzyme by directly modifying a subset of patients' own cells.[9] A large range of viral vectors have been trialed for this purpose. In the last decade or so, AAVs emerged as the most useful vectors for CNS-directed gene therapy due to their transduction efficiency, wide tropism, and relative safety profile. In particular, direct administration of small, non-enveloped, and non-integrating AAVs, named recombinant adeno-associated viral vectors (rAAVs), has been trialed both systemically and locally. A comprehensive overview of retroviral, lentiviral, and adenoviral-based vectors together with a discussion of their pros and cons for *in vivo* gene therapy and CNS targeting has been provided in recent reviews.[9,137,138] Here we focus on the most relevant pre-clinical and clinical data, specifically discussing how to increase AAV-mediated CNS-targeted expression.

Use of different AAV serotypes and capsids

One of the greatest advantages of rAAVs over other viral vectors is the possibility to choose different serotypes—for example, those with CNS-tropism can be utilized with the aim of improving *in vivo* gene therapy outcome for neurological LSD patients. Several *in vivo* studies showed that serotypes 5, 8, and 9, and the recombinant human (rh)10, can cross the BBB, each to a different extent, allowing transduction of the CNS following systemic administration.[139–142] For example, AAV9 was shown to be able to cross the BBB and improve neurological symptoms after systemic administration in LSD animal models.[143,144] Two open-label, dose-escalation, phase 1/2 global clinical trials assessing AAV9 technology via a single-dose i.v. infusion are currently underway for young (2 years old or less) and asymptomatic (development quotient >60) MPS IIIA (NCT02716246) and MPS IIIB (NCT03315182) patients, called ABO-102 and ABO-101, respectively. For the MPS IIIA trial, data collected at different time points (6, 12, and 24 months post-treatment) from the three dose-escalating groups highlighted a provisional safety profile in all patients, with time- and dose-dependent statistically significant reductions in cerebrospinal fluid and plasma heparan sulfate levels, and stabilization or improvement of adaptive behavior and/or cognitive function.[145,146] Another trial on MPS IIIA patients in middle and advanced phases of the disease receiving the highest dose of ABO-102 ($3 \times 10e13$ vg/kg) has recently terminated due to lack of efficacy (NCT04088734).[147] Preliminary results from the MPS IIIB trial were promising, with multiple disease biomarkers providing clear evidence of a biological effect in patients.[148]

Indeed, use of serotypes able to naturally target the CNS, such as AAV9, has been pivotal in providing access to the CNS. However, the low efficiency and lack of target specificity mean that high vector load needs to be used, potentially leading to toxicity. Generation of novel capsids would be important in increasing AAVs' specificity and efficiency. Years of capsid engineering efforts using different platforms have now yielded a number of improved CNS capsids for rodents that are undergoing pre-clinical testing.[149–151] In one recently published study, Chen et al. evolved a family of AAV capsid variants that can efficiently transduce both the central and peripheral nervous



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system in rodents. Both vectors also enable efficient targeting in non-human primates.[152]

Increased AAV dosing

Historically, serotypes AAV8 and AAV9 have preferential tropism for liver and muscle,[153] but, when used at higher doses, these serotypes might achieve more widespread tissue expression, including in the CNS. However, dose-related neurotoxicity has been reported in large animal models treated with high doses of AAV9.[154] Severe adverse events have been described in at least three clinical trials for other genetic disorders where high doses of the vector were administered, including increased serum transaminase (NCT03306277), complement activation and acute kidney injury (NCT03362502), and sepsis-induced deaths (NCT03199469).[155] These observations highlight the need to gather further safety data and, even when this has been obtained, these findings must be considered carefully because the severe immune response observed in these patients was not seen previously in animal models, making the outcome of this strategy to increase widespread tissue targeting unpredictable.[156]

Local delivery of AAVs

Local AAV delivery may allow BBB circumvention and enhanced delivery of therapeutics to the CNS. As described before for ERT and HSCT, there are several routes of administration to exploit (Figure 2) and the choice of one over another takes into account several factors, such as injection route difficulty and its prime therapeutic sites, the type of enzyme to express, cell type(s) to target, and their localization and distribution within the CNS. As direct CNS administration routes were discussed previously (sections "injection routes" and "administration routes") and have been extensively reviewed elsewhere,[157] only a few relevant examples are discussed here.

Preliminary results of clinical trials for CLN2 deficiency (Batten disease) pediatric patients based on intracerebral injection of AAV2 (NCT00151216)[158] or AAV2/rh10 (NCT01161576, NCT01035424, and NCT01414985)[159] have demonstrated a slower rate of gray matter loss and a significantly reduced rate of neurological decline, including motor and language function. Intracerebral administration of AAV2/rh10 and AAV2/5 has also been trialed for MPS IIIA (NCT01474343, NCT03612869)[160] and MPS IIIB (EudraCT 2012–000856-33),[161] respectively, showing moderate improvement in neuropsychological evaluations of behavior, attention, and sleep. Furthermore, a phase I/II clinical trial for intracerebral delivery of AAV2/rh10 for early onset MLD has reached completion and results should be available soon (NCT01801709). In several of the children treated in these clinical trials, a mild systemic immune response was observed,[159] while others presented with abnormal MRI results and experienced seizures,[159] or AAV vector was present in urine.[160] These observations perhaps suggest leakage from the CNS injection site into the periphery, triggering the immune response and hampering overall *in vivo* gene therapy efficacy. Transient immunosuppression by neonatal AAV-mediated systemic expression of a therapeutic gene prior to CNS-targeted *in vivo* gene therapy, and induction of liver-mediated tolerance,[142,156,162] have been trialed in MPS IIIA patients to address these concerns, with promising results.[160,163]

In terms of other injection routes, a small number of clinical trials based on i.t./i.c. administration of AAV9 serotype for MPS IIIA (EudraCT 2015–000359-26), MPS I (NCT03580083), and MPS II (NCT03566043) are currently underway. Only a small number of pre-clinical studies of i.c.v. injection have been conducted to date; pre-clinical studies in CLN2-deficient dogs with AAV2[164,165] showed delay of neurological progression and prolonged lifespan;[165] however, one animal experienced impaired cardiac function, likely due to augmented storage deposition in the heart.[164] In other pre-clinical studies performed in Niemann-Pick C,[166] MPS IIIA,[167] and MPS I mice,[166] animals treated i.c.v. with AAV2/9 showed reduced neurodegeneration, increased motor function, and extended lifespan.

Optimization of gene therapy cassette and AAV engineering

An indirect method to target the CNS is to engineer systemically delivered AAVs to produce enzymes that have an enhanced ability to cross the BBB. This can be achieved by including fusion proteins in the therapeutic construct (as discussed extensively in "enzyme modification"). Alternatively, the use of tissue-specific promoters, secreting peptides and optimized gene sequences can increase expression, secretion, and uptake of the therapeutic enzyme respectively.[168] This strategy might also overcome the limitation of using serotypes that have restricted CNS tropism. In addition, bioinformatics-guided design of lysosomal enzymes may not only improve enzyme production/secretion/uptake, but also reduce immunogenicity.[169]

CONCLUSIONS AND FUTURE PERSPECTIVES

Here, we have explored strategies to increase the ability of enzymes, stem cells, or viral particles to engraft the damaged CNS of neurological LSD patients (Figure 4).

While choosing the appropriate therapy for each LSD is of vital importance, timing of the intervention is almost as critical. Treatments administered when patients are still asymptomatic have proven to be more effective in both animal models[170–172] and patients,[173–177]

Figure 4. Summary of the different strategies used to improve delivery of therapeutic agents to the CNS in the treatment of LSDs

(A) Blood-brain barrier (BBB) disruption strategies: (I) ultrasound or (II) hyperosmotic agents can be used to disrupt the integrity of the BBB; (III) stimulation of receptors can increase passage of enzymes and/or stem cells across the BBB. (B) Enzyme replacement therapy can be targeted to the CNS by modifying enzymes directly (I) or using delivery vehicles to facilitate easier passage across the BBB (II). A range of delivery methods (III) including convection-enhanced delivery, direct injection routes, and delivery vehicles can be used to target the CNS. (C) *Ex vivo* genetic modification of stem cells using gene therapy (I), different pre-conditioning regimes/agents (II), and different injection routes (III) have been trialled to improve CNS targeting in stem and progenitor cell transplantation. (D) Modifications of gene therapy constructs, including optimization of the gene cassette (I) and selection of viral serotype with CNS tropism (II), and specific injection routes (III) can be utilized to target the CNS with *in vivo* gene therapy approaches

highlighting the need for early intervention and implementation of newborn screening for more LSDs. In this direction, *in utero* intervention may circumvent the BBB selectivity issue, as at this developmental stage the BBB is not yet functional; moreover, transplanted cells can engraft and occupy the microglial niche during the same developmental time frame as resident cells,[178] removing the need for pre-conditioning. To this end, a pre-clinical study in MPS VII mice showed that *in utero* delivery of ERT or HSCT improved neurological symptoms.[178] *In utero* HSCT has been successfully applied in small-scale clinical trials for severe combined immunodeficiency patients, and less successfully for thalassemia patients.[179] However, a careful benefit/risk assessment of *in utero* procedures must be performed and further pre-clinical and clinical studies would be required to support routine application.

Among all the strategies described here, BBB manipulation techniques are relatively easy and cheap compared with others; however, they provide non-selective permeability, posing the risk of toxicity.[24] For clinical application to be a realistic prospect, toxicity must be limited, and patients would require strict monitoring for adverse events.

Immunogenicity of therapeutics needs to be considered carefully too, as this can trigger the immune system and subsequently reduce treatment efficacy. In the case of ERT, the repeated infusion of enzyme often results in immune reaction against the enzyme itself[64] and furthermore negatively affects therapeutic impact in the CNS; following ERT in an MPS I canine model, animals with a high titer of antibody against the therapeutic enzyme showed less-significant reduction of storage product accumulation in the brain than those with lower antibody titers.[180] Similarly, despite rAAVs for *in vivo* gene therapy having several advantages over other viral vectors, including relatively low immunogenicity,[181] long-term gene expression,[182,183] and wider tissue tropism, they still trigger the immune system; T cell responses to the transgene might appear after AAV-based vector administration.[184,185] Moreover, as the majority of humans have already been exposed to several wild-type AAV serotypes, neutralizing anti-capsid antibodies might be either present in patients prior to treatment[186–188] or arise quickly following the first administration, rendering vector re-administration not a viable option.[189] Continued efforts to minimize the immunogenicity of all therapeutics is vital to the success of ERT and gene therapy, especially in the CNS where prolonged inflammation can have particularly severe negative consequences, as shown, for example, in the case of viral encephalitis, in Alzheimer's patients, and in association with diabetes.[18,19,21–23]

Another important point is the need for efficient targeting. A notable disadvantage of rAAVs for CNS-targeted gene therapy is that they can only efficiently transduce neurons, and no other disease-relevant brain cells, such as microglia, oligodendrocytes, or astrocytes.[140,190] However, not all cells must be corrected for treatment to be able to exert therapeutic effect due to so called "cross-correction," especially if therapeutics have been modified to deliver supraphysiological levels of enzyme.

Among the most successful and safe strategies for neurological LSDs is HSCT gene therapy. In the last 2 years, two medicinal products based on HSC gene therapy strategies have been approved in Europe; Libmeldy[129] for MLD and Skysona[191] for ALD (NCT01896102, NCT03852498, NCT02698579). Skysona also received accelerated FDA approval in September 2022.[192] This has brought great enthusiasm and renewed hope to neurological LSD patients.

A further consideration for wide adoption of these single administration gene therapies in healthcare systems is pricing and reimbursement. Current models of payment for chronic therapies, such as ERT, accept regular costs year on year for the lifetime of an individual; the cumulative costs of which can be considerable with a recent cost-analysis estimate between €9.3 and €9.7 million (£8.1 and £8.5 million) for LSD treatment.[193] This needs to be balanced against a once off payment for single-administration cell and gene therapies, where, although the initial price may be considerable, this is deemed appropriate given the long-term overall clinical benefit.[194,195]

Even though HSC gene therapy holds great potential, one main issue remains for its clinical suitability, namely the suboptimal, and in some case minimal, engraftment of HSCs in the CNS. The goal is to engraft a sufficient number of HSC-derived cells able to differentiate into microglia and act as a constant and never-ending source of enzyme secretion. A crucial role for a successful CNS engraftment is played by the conditioning regime chosen to clear the niche (by depletion of the native microglia) for donor HSCs. Engraftment in the CNS is significantly improved by busulfan conditioning compared with irradiation,[102] with busulfan being the regime of choice for neurological LSD patients[196] before transplantation. However, busulfan is associated with a substantial systemic toxicity,[108] and alternative strategies based on CNS-targeted microglial depletion may represent less toxic and safer pre-conditioning alternatives for neurological LSD patients in the longer term.

Another way to increase CNS engraftment would be to focus on improving stem cells' crossing of the BBB; studies aiming to understand what HSC subpopulation engraft the CNS and the mechanisms they use to cross the BBB would be helpful in devising new strategies to increase BBB cell permeability.

At the moment, no single therapeutic approach discussed here provides the perfect solution for every neurological LSD,[197] supporting the idea that, for these neurometabolic disorders, the CNS component remains a significant challenge. However, in these monogenic severe disorders, where there is a clear genetic component and pathway to be addressed, there is a unique opportunity to develop therapeutics that can have significant impact and which, if successful, may have wider application to more common forms of neurodegeneration.

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AUTHOR CONTRIBUTIONS

B.J.C. wrote the manuscript's draft under the supervision of H.B.G. and S.B. S.B. coordinated the work, contributed to the draft, finalized the manuscript, and acquired funding.

DECLARATION OF INTERESTS

H.B.G. is the CEO of Orchard Therapeutics. The other authors declare no competing interests.

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