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Lysosomal storage disease: gene therapy on both sides of the blood-brain barrier

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Abstract

Most lysosomal storage disorders affect the nervous system as well as other tissues and organs of the body. Previously, the complexities of these diseases, particularly in treating neurologic abnormalities, were too great to surmount. However, based on recent developments there are realistic expectations that effective therapies are coming soon. Gene therapy offers the possibility of affordable, comprehensive treatment associated with these diseases currently not provided by standards of care. With a focus on correction of neurologic disease by systemic gene therapy of mucopolysaccharidoses types I and IIIA, we review some of the major recent advances in viral and non-viral vectors, methods of their delivery and strategies leading to correction of both the nervous and somatic tissues as well as evaluation of functional correction of neurologic manifestations in animal models. We discuss two questions: what systemic gene therapy strategies work best for correction of both somatic and neurologic abnormalities in a lysosomal storage disorder and is there evidence that targeting peripheral tissues (e.g., in the liver) has a future for ameliorating neurologic disease in patients?

Keywords

behavior; CNS; neurologic; mucopolysaccharidosis; *Sleeping Beauty* transposon; transcytosis

1. Introduction

1.1. Lysosomal Storage Diseases (LSD) and their therapies

LSD comprise inherited monogenic diseases caused by deficiency of one or more lysosomal enzymes [1]. Enzyme deficiency results in progressive intra-lysosomal dysfunction

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characterized by the accumulation of uncleaved lipids, glycoproteins and/or glycosaminoglycans that lead to secondary accumulation of other macromolecules [2]. The consequence is alteration of cell morphology, impaired autophagy, oxidative stress and neuroinflammation, which in turn lead to impaired function of organs and tissues [3-8].

One particular class of LSD is the mucopolysaccharidoses (**MPS**) in which the enzymes that degrade glycosaminoglycans (polymeric sugar-carbohydrate chains) are defective. MPS disease occurs in about 1/25,000 births. LSD, and MPS in particular, are complex disorders with symptoms that affect most organs of the body, including the central nervous system (**CNS**), Current therapies, comprising enzyme replacement therapy (**ERT**) and hematopoietic stem cell transplantation (**HSCT**), are some of the most expensive in medicine. ERT varies from $100,000 to $500,000 per year and a single round of HSCT costs about $200,000 [9]. Long-term effectiveness of these expensive therapies is not clear either; indeed, studies have shown that there is a statistically significant association between duration of ERT use and worsening quality of life [10]. Gene therapy offers the possibility of affordable, comprehensive treatment of all of the problems associated with these diseases. It took almost two decades of research to appreciate the complexities of LSD and MPS that have to be surmounted for gene therapy. Nevertheless, based on recent developments, there are realistic expectations that effective therapies may be coming soon. Here we discuss some of the recent advances, since the earlier comprehensive reviews [11-15].

Treatments of MPS and many LSD are generally based on the phenomenon of *cross-correction* [16], which is the ability of lysosomal enzyme-expressing cells to correct others that are enzyme-deficient. This *bystander effect* is possible because approximately 10% of the lysosomal enzymes manufactured in a cell will naturally escape into the circulation for recapture by other cells. Circulating lysosomal enzymes are taken up by cells via mannose-6-phosphate (**M6P**) or mannose receptors, a process called *receptor-mediated endocytosis* [17]. Thus, for therapy only a relatively small number of cells expressing the missing lysosomal enzyme is required to correct many other cells that are unable to produce enzyme [18]. Besides receptor-mediated endocytosis, cross-correction depends on the efficiency of secretion of a lysosomal enzyme from the cell in which it is made, which is strongly determined by the signal peptide associated with the enzyme [19].

ERT and bone marrow transplantation (**BMT**)/autologous HSCT are the two clinically available therapies for LSD. ERT, in which a purified recombinant enzyme is infused into the patient for amelioration of somatic disease [18], is inefficient for treatment of the neurologic disease because the intravenously administered enzyme does not transit the blood-brain barrier (**BBB**) [20]. Transient disruption of the BBB with hyperosmotic solutions can be performed in the clinic, but repeated opening of the BBB can injure the brain [21].

HSC therapy on the other hand has a potential to ameliorate CNS-related deficits [22-24] because, following infusion of bone marrow-derived cells from a matched donor, monocytes that circulate in the blood can engraft the CNS as either perivascular or meningeal macrophages [25-27] Alas, the natural lysosomal enzyme activity in HSCs is too low to be cross-corrective for most LSD. But, HSC can be genetically modified using integrating gene

therapy vectors to overexpress the therapeutic enzyme [25, 28] and lentiviral vectors have proved to be particularly effective for the purpose [29-31]. This strategy has been successfully employed in the first human clinical trial for metachromatic leukodystrophy, a prototypical LSD [32]. However, even in experienced centers HSC transplantation still has a mortality rate of at least 10% [33, 34].

Accordingly, there remains a need to develop non-invasive and relatively simple therapies to correct LSD-related deficits in somatic and nervous tissues. Gene therapy is the most promising type of therapy because some viral vectors are capable of transducing a broad spectrum of tissues after a single systemic administration [35]. However, viral vectors have drawbacks that include their expense and issues related to manufacturing; hence, non-viral vectors would be preferable [36]. On the other hand, the major problem with non-viral vectors is their inability to enter cells except under extraordinary conditions and their inability to penetrate the BBB. Thus, the challenge is clear – to develop a therapy that is affordable, reliable, and capable of treating all tissues of the body. This therapy should be durable such that one or very few treatments will last a lifetime. In the following sections we describe candidate vectors and approaches for gene therapy that have the potential of meeting these goals. But first we describe the BBB that is the major obstacle to effective gene therapy because it limits passage of corrective enzyme from the blood circulation to the neural tissues.

1.2. Structure and function of the BBB

The structure, function and breaching of the BBB for delivery of therapeutics to the CNS have been extensively reviewed [21, 37-40], including gene therapy for LSD [40-42]. The core anatomical element of the BBB is the network of cerebral blood vessels formed by endothelial cells and supporting cells such as astrocytes, pericytes, neurons, and perivascular microglia [21]. The blood capillaries of the BBB are typically within 8-25 μm of each neuron. Endothelial cells of the BBB are unique among other endothelial cells in their continuous intercellular tight junctions (two adjacent cells with membranes that appear to be fused), lack of fenestrations and extremely low rates of vesicular transport. All of these features greatly limit the movement of molecules through the endothelial layer. This physical barrier significantly reduces permeation of ions and small hydrophilic molecules. As a result, passage of molecules *through* cells via transcellular pathways can be easier than *between* cells [40]. There is a related barrier between the circulation and the spinal cord, the blood–cerebrospinal fluid (**CSF**) barrier in which tight junctions are formed between the epithelial cells of choroid plexus and the ependymal lining of the brain ventricles that secrete CSF. The ventricles are interconnected and are connected with the central canal of spinal cord, allowing the flow of CSF. There are also several other specialized neural barriers in specific enervated tissues such as the blood–retinal barrier and the blood–labyrinth barriers [21].

Active uptake and transport of proteins across the endothelial and epithelial cell layers occurs via two coupled processes, receptor-mediated endocytosis and transcytosis [43]. Although poorly understood, transcytosis is the principal vesicular mechanism by which large molecules can move across the brain endothelium [40]. M6P receptors on the BBB are

down-regulated in the course of development, e.g., in mice by two weeks after birth [44, 45], but receptors for important brain feeders are active and transport specific peptides and large molecules across the BBB. Innovative strategies developed over the past decade have focused on constructing fusion proteins with either antibodies or ligands to bind to those receptors and thereby "bootleg" their cargo into the brain [18, 21, 46, 47]. Receptors include the low-density lipoprotein receptors that bind apolipoproteins (Apo) B and E. For lysosomal enzymes, feasibility of this brain-targeting approach was first demonstrated in a proof-of-principle study by Spencer and Verma [48] in which lentivirus-mediated fusion glucocerebrosidase supplied with ligands to these receptors was detected in lysosomes of neurons and astrocytes of the mouse brain following a single intraperitoneal vector injection. Repeated high-dose treatment with a therapeutic enzyme into the CSF as a way of direct delivery to the CNS can be effective in reducing neuropathology in MPS IIIA mice [49].

Passive entrance of molecules into the brain from the circulation also occurs, depending on such molecular characteristics as size, charge, lipophilicity, etc., and is proportional to concentration in plasma [50, 51]. Vogler et al. [52] were the first to demonstrate breaching the BBB with prolonged exposure to the circulating lysosomal enzyme β-glucuronidase supplied at doses higher than those conventionally used for ERT. As a result, in a murine model of MPS VII there was a dose-dependent amelioration of CNS pathology following repeated ERT. Their conclusions have been since supported by studies in many labs [53-60]. These findings are particularly relevant to liver-directed gene therapy because gene therapy of systemic diseases, viral and non-viral, generally relies on creating an *enzyme depot*. Gene-modified liver can support the manufacture of relatively large amounts of corrective enzyme that can be distributed via the circulation throughout the body.

The challenge is to find either viral or non-viral vectors that can circumvent the BBB for effective gene therapy on both sides of the BBB. Taking the examples of MPS I and MPS IIIA as model diseases, we review strategies that employ non-viral and viral vectors administered into the bloodstream or CSF, as well as progress using virally transduced HSCs for treatment of LSD, in particular MPS.

1.3 Non-viral gene therapy

Two effective methods for non-viral gene therapy have been developed in mice. The first is transposons, exemplified by the *Sleeping Beauty* **(SB)** transposon system that support sustained, supraphysiologic levels of transgene expression in mammalian cells [61-63] and have been effective for gene therapy of several systemic diseases in mice, including lysosomal storage disorders MPS I and MPS VII [54, 64]. The second is minicircles, circular DNA molecules derived from plasmids following the removal of nearly all plasmid "backbone" sequences to leave only the transgenic expression cassette [65, 66]. Minicircles appear to support sustained, extrachromosomal activity from episomes, which is considered advantageous for gene therapy because it avoids issues associated with insertional mutagenesis. The disadvantage of minicircles is that when cells divide, episomes will not necessarily be carried to the daughter cells. Minicircles have been used to treat MPS I in mice [67]. However, despite considerable progress in non-viral amelioration of LSD-related problems in many organs and tissues in mice, correction of CNS impairments remains a

problem because non-viral vectors cannot effectively transduce cells in the CNS, whether administered directly or systemically.

For achievement of the systemic therapeutic effect, supraphysiologic activities in the mouse serum are needed, in some cases as high as 100-fold wild-type (**WT**) levels, e.g. of α-L-iduronidase for MPS I [53, 54] (**Table I**), which can be attained by *hydrodynamic injection*. This delivery procedure is highly effective in mice; a volume equivalent to 10% weight of the recipient mouse is infused through the tail vein in less than nine seconds [68-70]. Although systemically delivered, more than 99% of the transgene expression is in the liver [71]. Stable supranormal concentrations of the therapeutic enzyme in serum might ameliorate the nervous system in a dose-dependent way [52] [52]. We will return to this point.

1.4. Virus-mediated gene therapy

Gamma-retroviruses (**RV**) were the first vectors used for gene therapy of LSD [11, 14, 72, 73]. The earliest vectors were based on simple murine leukemia virus genomes but as issues with insertional mutagenesis were appreciated, self-inactivating retroviruses (**SIN-RV**) were designed as potentially safer backbones [74, 75]. Although SIN-RV yield lower levels of transduction and transgene expression compared to the original **LTR-RV** and are, therefore, less efficient in correcting clinical manifestations, this might be overcome by using higher doses of these vectors. As described in detail below, RV vectors can correct some CNS deficits even though they do not cross the BBB.

Direct delivery into the brain is possible for viral gene therapy. Intraparenchymal stereotaxic injections, which are invasive and allow injections of only very small volumes of fluid (e.g., in a mouse <10μl/injection), result in localized gene expression, are problematic for scaling up to the human brain and do not ameliorate peripheral nervous system manifestations (**PNS**) [76-78]. Direct delivery to the CSF is an alternative route of vector administration that can be performed in animals into cisterna magna or via lumbar injection. However, the latter route is inefficient in large animal models, while the former route is avoided in pediatric clinical practice due to the smaller size of cisterna magna and the danger of injury to vital centers [77, 79].

For delivery of transgenes to the CNS, adeno-associated virus (**AAV**) vectors appear to be the most effective. Some serotypes, such as AAV9 family of vectors, can cross the BBB and transduce endothelial cells, neurons and astrocytes throughout the CNS and PNS, as well as somatic tissues after a single administration into either the blood or the CSF [76, 80-85]. Therapeutic effect using AAV9 has been demonstrated in mice [76, 79, 83], cats [78, 82, 86], dogs [78, 87-89], pigs [90] and non-human primates [35, 83, 91, 92]. AAV9 vectors also may be efficient in correcting the CNS following intranasal delivery (Lalitha Belur and Scott McIvor, personal communication), an alternative non-invasive route into the brain.

While the BBB-crossing AAV vectors (e.g., serotypes 9, rh.10, etc.) via *in vivo* delivery outperform all other AAV serotypes and other vectors in global correction of systemic disease with neurologic involvement, they share drawbacks of all viral vectors that include the following: the high cost of delivery, variable efficiencies of production and storage, as

well as immunogenicity. In addition, gender bias has been reported for liver-expressed AAV vectors in mice and dogs [79, 93-96], whereby 2-13-fold higher activities of transgenic products were measured in the liver and serum of males following intravenous treatment with AAV serotypes 2, 5, 8 and 9. This gender effect, presumed to be androgen-driven [93], was observed in immunocompetent and immunodeficient animals. Gender bias appears promoter-independent, e.g., the ubiquitous CAG (CAGGS), as well as the liver-specific hAAT promoter (**Table I**). Notably, delivery of AAV9 into the CSF had similar effect on liver expression but CNS was not affected [79]. Of note, our experience with SB transposon-mediated liver-directed gene therapy showed no gender bias when the same liver-specific promoter was used; however, with the ubiquitous CAGGS, human IDUA expression in the liver and activity in the serum was up to 50-fold higher in males than in females, both in immunodeficient NOD.129(B6)-*Prkdcscid IDUAtm1Clk* and immunocompetent C57BL/6 mice. The extent of male vs female difference in IDUA activities was dependent on the SB transposon dose [54, 97]. On the other hand, Maguire et al. [98] reported a 2-3 –fold higher transduction and consequent transgene expression in female mouse brains upon intravenous injection of AAV9 into mice of two different strains, nude and C57BL/6. These contradictory findings emphasize how poorly understood the mechanisms underlying gender bias in gene therapy are and the importance of treating animals of both genders in pre-clinical gene therapy studies [54, 95].

A big issue in transition to clinical trials is immune responses. Following intravenous delivery, systemic exposure to high doses of AAV vectors can trigger the activation of $CD8^+$ T-cell responses directed against the viral capsid in a dose-dependent manner [99-102]. Neutralizing antibodies to either the viral capsid [103] or the expressed therapeutic protein may block transduction of peripheral organs, although are not necessarily associated with adverse clinical consequences or loss of efficacy, particularly, when “self” enzyme is expressed [78, 86, 104, 105]. Because AAV9 can transduce antigen-presenting cells regardless of the route of administration, these vectors also trigger antigen-specific immune responses that can eliminate expression of the therapeutic protein [79, 105]. Thus, with viral vectors there is a tradeoff of effective delivery with immune responses at several levels that diminish the effectiveness of treatment.

1.5. Virus-modified HSC therapy

Gene therapy-modified HSC combine advantages of an autologous source of hematopoietic stem cells that avoid immune response issues with the benefits of enzyme overexpression from integrating viral vectors in transplanted cells [25, 28-30]. Lentiviral vectors (**LV**) appear to be most efficient for transducing HSC (**LV-HSC**). The Biffi group [29, 106] demonstrated progressive and extensive reconstitution of well-differentiated microglia in the CNS by the transgene-expressing progeny of transplanted hematopoietic progenitor cells in mice with the prototypical LSD metachromatic leukodystrophy. The first clinical trial showed LVHSC-mediated prevention of the development of major disease manifestations in children treated at the presymptomatic stage [32]. This strategy may soon become a new therapy.

2. Circumventing the BBB for treatment of MPS I and MPS IIIA

To appreciate various experimental systemic strategies to breach the BBB for whole-body treatment of LSD, we focused on mouse studies for two types of LSD - MPS I and MPS IIIA, which are caused by the deficiency of either α-L-iduronidase (**IDUA;** EC 3.2.1.76), or sulfamidase (**SGSH;** EC 3.10.1.1), respectively. Both MPS I and IIIA have well-characterized mouse models with phenotypes resembling the human diseases [3, 28, 107-111]. In all the studies reviewed here, mice were treated as young adults up to three months of age with a single systemic administration of the therapeutic agent. IDUA is required for degradation of glycosaminoglycans (**GAG**), dermatan sulfate and heparan sulfate (**HS**). Besides the nervous system, many organs are affected in IDUA-deficient animals and humans with the severe form of MPS I. Deficiency of SGSH causes accumulation of HS that results in cellular and organ dysfunction, which is particularly severe in the brain and leads to profound mental retardation and neurodegeneration [3] as well as a significantly shortened lifespan. Global neuroinflammation is a signature trait in MPS IIIA. Behavioral changes are severe and include hyperactivity and decline of cognitive and motor functions [3, 109, 111]. Although both MPS I and MPS IIIA require global restoration of the missing enzyme activity, gene therapy effective for neurologic disease is of particular importance in MPS IIIA, but clinically available treatments are ineffective: ERT does not cross the BBB, while BMT does not produce a sufficient, therapeutic level SGSH [110, 112].

2.1. Correction of defects in MPS I mice by liver-directed and LV-HSC gene therapy

Table I summarizes the use of *in vivo* and *ex vivo* gene therapies for treatment of MPS I in mice. RV vectors of two different designs ("Viral approaches with unmodified IDUA" in **Table I**) yielded transgenic activity levels in plasma 25- to 84-fold of WT, about the same levels of correction as transposons and minicircles ("Non-viral approaches with unmodified IDUA" in **Table I)**, i.e., 70-100-fold above WT level. The table also shows that plasma/brain relative IDUA activity ratios were about 100-fold for both viral and non-viral strategies that involve transgene expression restricted to somatic tissues and supraphysiologic activity in the blood. Most importantly, in hindsight, it is clear that there is no accepted standard for evaluation of effectiveness in CNS correction. For example, IDUA activity in the brain is reported for either total brain tissue or parts of the brain. Whereas some studies focused on reduction of lysosomal pathology and/or GAG levels in whole brain homogenates others looked at specific neural cells, which is important for translating biochemical information to behavioral consequences.

The few entries in **Table II** compared to **Table I** testify that most investigators have focused on biochemical benchmarks compared to behavioral correction. Behavioral evaluation of CNS correction in the RV study showed some level of functional correction with both LTR- and SIN-RV, which was better with higher serum IDUA levels. While all mice somewhat improved in habituation and horizontal locomotion, only mice with plasma IDUA activities of about 80-fold WT significantly differed from MPS I mice in sensorimotor performance (**Table II**). The data in **Table II** suggest that even with restoration of IDUA levels to about 70% in the forebrains of treated mice not all behavioral activities, such as rearing and

running up the walls of the activity chamber, were restored [113]. This may reflect either an inability to reverse the pathology that occurred before treatment, or unequal correction in different parts of the nervous system [76, 114]. Clearly, future evaluation of behavioral correction following delivery of nonviral vectors, which attain the same levels of biochemical correction in the blood, must include a panoply of tests in order to allow comparisons with viral therapies.

Compared to liver-directed gene therapy approaches, *ex vivo* lentiviral modification of HSCs can be expected to result in better CNS correction because transgenic enzyme in the brain comes not only from the circulation but also from repopulating microglia within the brain itself [25, 29, 106]. Visigalli et al. [115] attained IDUA activities of 2-4.5X WT in the brains of all LV-HSC-treated MPS I mice, which correlated with IDUA activity in peripheral blood mononuclear cells and plasma of about 100–fold WT. Thus, as shown in **Table I**, viral and nonviral treatments of just the liver that supported a 100X WT level of serum IDUA led to only about 6% WT of whole brain activity, whereas LV-HSC treatment resulted in about the same level of serum IDUA (100-200X) but a brain IDUA level of 200-500% WT. That is, LV-HSC was about 100-fold more effective in treating the brain than with just gene transfer to the liver. Examination of adaptive behavior and memory deficits in repeated open-field tests showed normalization of both vertical (rearing), and horizontal locomotor activities following LV-HSC treatment (**Table II**) but sensorimotor tests that would permit comparison to the extent of functional correction with RV were not conducted.

Inner ear and retina are separated from the circulation by specialized neural barriers. Accumulation of GAG has been observed within the cells of the inner ear of MPS I mice [75, 113, 116, 117]. Both LV-treated HSC [115] and high-expressing RV [75, 113] led to significant improvements in hearing (**Tables I** and **II**). However, neither treatment completely corrected auditory-evoked brainstem response. Histopathology examination performed in the RV studies [75, 113] suggested that improved hearing was likely due to marked reduction of lysosomal storage in the round window membrane. However, a middle ear exudate was consistently present even in mice treated with the highest IDUA activities, likely due to lack of efficient access of IDUA to the middle ear. Improvements on motor coordination tests found in LV-treated mice with high activity serum IDUA suggested correction of vestibular function, although interpretation of motor-coordination results was confounded by improvements in bone and joint disease and/or muscle strength, all of which could play a role in the amelioration.

Ocular disease was corrected in both high-activity RV- and LV-HSC-treated mice in terms of improvement of the photoreceptor count in the retina [74] and restoration of retinal thickness [115], respectively. The above data strongly suggest that the low activity in the brain resulting from passive diffusion of IDUA can support some functional gain and that the degree of correction strongly depends on availability of the enzyme in the affected tissues, which correlates with the dose of the enzyme circulating in the blood. The data in **Tables I** and **II** also suggest that to achieve activity levels of IDUA in the brain comparable to those attained with LV-HSC, liver-directed gene transfer, non-viral or viral, would have to produce about 5000-fold higher enzyme activity than WT, unless a method of specific breaching of the BBB by therapeutic enzyme is developed. Our experience with increasing

plasmid dose of IDUA-encoding SB transposons showed that above a certain level, there is no further proportional increase in sustained activity [97]. Recently, Chuah et al. [118] reported rationally designed transposon vectors that can direct elevated levels of gene expression after hydrodynamic delivery to the liver in mice. In these vectors appropriate assembly of transcriptional regulatory motifs is coupled with modifications of the transgene and the vector. These improvements suggest that further increase of transgene expression 10- to 100-fold may be possible, which would significantly increase the prospects for successful DNA-based, liver-directed gene therapy.

2.2. Correction of defects in MPS IIIA mice by liver-directed and LV-HSC gene therapy

Direct correlation between activity of therapeutic protein in plasma and its neurologic effects has also been demonstrated in the MPS IIIA mouse model. Generally, liver-based viral therapy is particularly ineffective for CNS correction for this disease, probably because of low secretion of the SGSH enzyme out of hepatocytes [119]. However, using AAV8 vectors, Ruzo et al. [96] attained 10% WT SGSH activity in the brains of MPS IIIA mice when the sustained plasma activity of SGSH was about four-fold WT (**Table III**), which was sufficient to reduce substantially brain histopathology. Because in MPS IIIA survival correlates with the degree of neurodegeneration, prolonged lifespan of treated mice supports the argument that the transgenic SGSH made in the liver was functional after crossing the BBB. Notably, due to a gender bias in liver-directed AAV therapy, the correction was attained only in the male mice whose activity in plasma was several times higher than that in females.

As was the case with treatment of MPS I, a higher activity of transgenic SGSH in the mouse brain was achieved with LV-HSC [120]. This group focused on increasing SGSH expression in monocytes/microglia cells by using the myeloid-specific promoter CD11b rather than the ubiquitous PGK [115] to drive expression of SGSH. Moreover, they improved repopulation of microglia in the brain and “codon-optimized” the human SGSH. The net result of all of these adjustments was an 11% WT activity in the brain (**Table III**), which was sufficient to correct lysosomal distention and neuroinflammation as well as improve behavior as reflected by open-field testing (**Table IV**).

2.3. Correction of defects in MPS IIIA mice by AAV9 intrathecal gene therapy

Direct infusion of BBB-crossing AAV9 into the CSF of MPS IIIA mice [79] led to SGSH on *both* sides of the BBB with significant reduction of GAG in the brain (**Table III**) and commensurate restoration of selected behavioral activities (**Table IV**) in a dose-dependent manner. With up to a 20-fold lower dose of vector, the levels of enzyme in the brain exceeded those achieved with the liver-targeted AAV8 or intravenous AAV9 treatments (**Table III**) and were reflected in the behavioral outcomes (**Table IV)**. Dissemination of the vector created an enzyme depot in the liver, which was the main source of the circulating enzyme that had corrective effect on multiple somatic tissues. Thus, passage of SGSH from the brain to the rest of the body is far more effective than from the blood circulation to the brain. A very important finding in the Haurigot study is that some parts of the brain, which were only partially corrected at 4 months, appeared fully corrected at 12 months post-

treatment. This observation suggests for the first time that persistent low-level expression can be curative in cases where only partial correction is observed with ERT.

Overall, the data in **Tables I** and **III** suggest that the BBB is not impenetrable but that the efficiency of passage of MPS enzymes through it is extremely low, at least from the peripheral system to the brain. This has spurred efforts to develop methods for selective breaching of the BBB to enhance gene therapy for LSD.

2.4. Fusion proteins to augment crossing the BBB

Active transport of transgenic proteins to the CNS has been explored by fusing transgenes to sequences that encode ligands to active receptors on the BBB [46-48]. Wang et al. [121] applied this idea by juxtaposing an IDUA cDNA to a sequence encoding a portion of the ApoE-binding domain for the low-density lipoprotein receptor LRP1, which is ubiquitous in the brain [43]. They tested targeting of the fusion protein encoded in a plasmid and LV-HSCs ("Fusion IDUAApoE" in **Table I**). In the latter case, the fusion-IDUA gene was placed behind an ankyrin-1 promoter, which is active only in maturing erythrocytes and thereby restricts IDUA expression to the blood while the plasmids, delivered by hydrodynamic infusions had a liver-specific promoter to direct expression in that organ. Compared to SB-transposons encoding unmodified IDUA in which IDUA activity in the brain to plasma ratio was about 1%, the ratio of fusion IDUA appeared to be about 5-10% that in the blood, i.e., about a 5-10-fold increase in efficiency of breaching of the BBB with LV-HSC infusions (**Table I**). Notable GAG clearance was also achieved. However, due to the weak ankyrin-1 promoter, the overall level of IDUA activity in plasma was low – below the "low-dose" treatment in the RV study, which likely contributed to the overall low activity in the brain. Alas, behavioral studies for fusion IDUA, which would clarify conclusions on the ultimate success of brain targeting, were not reported.

Sorrentino et al. [119] used fusion SGSH to attain brain targeting via liver-directed AAV gene therapy. Two ligands were added to the SGSH transgene - 1) the ApoB-binding domain to target the LDL receptor and 2) the signal peptide (**SP**) sequence from the iduronate-2-sulfatase enzyme to increase secretion. Comparison of liver-directed treatments using unmodified SGSH in AAV8 and the fusion SGSH mediated by AAV2/8 shows similar levels of hepatic expression in mice, even though the administered dose of the AAV2/8 virus encoding the fusion SGSH was 10 times lower (Table III). In the brain enzyme activities also attained similar levels (10%WT SGSH activity with AAV8 versus 13% activity in heterozygous brain with the fusion AAV2/8). Curiously, in serum activity of the unmodified murine SGSH was several-fold higher than that of the fusion human SGSH. There may be at least two explanations for this. First, the unmodified murine SGSH did not elicit immune responses that might have occurred with the human liganded-SGSH. Second, there may have been gender effects; while Ruzo et al. [96] found significantly higher activity in males than in females, gender bias in liver and serum activities were not reported in the fusion-SGSH [119] study; if mice of both genders were analyzed for SGSH activity as a pool the average would be lower in the case of gender difference (**Table III**). Nevertheless, the behavioral corrections following treatment with fusion SGSH (**Table IV**) correlated with the increases in enzyme activity in the brain.

Overall, the data in **Tables III** and **IV** are perplexing and underscore the need for a greater appreciation for more detailed analyses of outcomes at the biochemical and functional levels in future experiments. Specifically, Sorrentino et al. [119] and Sergijenko et al. [120] reported that 7-11% WT SGSH activity in the brain was sufficient for demonstrable behavioral correction, assuming that the 13% heterozygous levels translates to 7-8% homozygous WT level. However, Ruzo et al. [96] found no behavioral improvement with an indistinguishable 10% WT level in the brain. This suggests that tests used to evaluate neurobehavioral correction in different labs are not uniform and consequently support different conclusions on the efficacy of similar treatments.

In the fusion-protein studies above, a BBB-penetrating ligand was fused to the enzyme in a *cis* configuration. Since fusion can render some proteins inactive, an alternative approach has been proposed. Sarkar et al. [122] have shown that passage into the brain from the bloodstream can be achieved by delivery of the recombinant protein in *trans* with a receptor-binding ligand. This strategy appears to have been effective for treatment of murine late infantile neuronal ceroid lipofuscinosis, a neurodegenerative LSD caused by loss of tripeptidyl peptidase I (TPP1) [123]. A single intravenous co-delivery of TPP1 with a peptide that comprised polylysine and an ApoE receptor-binding segment led to restoration of TPP1 enzyme activity to about 400% WT and consequent significant reduction of lysosomal storage within the brain, as well as increased lifespan and improved neurological function. Whether or not this approach can be adapted to experimental gene therapy has yet to be seen.

The potential to use transcytosis for transport of chimeric proteins across the BBB is supported by the growing number of studies, but not by the number of clinical trials [51, 124] and there are issues that need to be addressed [37, 40, 51, 125]. These include the notion of the maximal transport capacity of the natural receptor-mediated transcytosis pathways in the brain, the potential for adverse effects due to competition between the natural and the engineered macromolecules as well as variability of expression levels of receptors on the BBB that are engaged in transcytosis.

3. Safety of enzyme overexpression

Overexpression required to achieve therapeutic threshold has shown no adverse effects in several murine LSD studies, including MPS I [115], MPS IIIA [120], MPS VII [126] and in pre-clinical studies and clinical trials for metachromatic leukodystrophy [32, 127]. While there is no evidence that overexpression of either IDUA or SGSH is harmful, some argue that restricting expression to desirable cell types provides an extra level of safety [120, 121]. For example, in the mouse model of Krabbe disease, GALC expression was toxic to HSC [128]. However, the overall efficiency of therapy can be enhanced by the appropriate choice of the transcriptional motifs driving transgene expression. For instance, the myeloid-specific CD11b promoter increased therapeutic expression in the brain [120]; other approaches restricted expression of IDUA to maturing red blood cells by using a hybrid human ankyrin-1 promoter to avoid overexpression in myeloid progenitors and thereby avoid activating proto-oncogenes [121]. However, this potential safety measure was at the expense of the expression level because the erythroid-specific ankyrin-1 promoter was too weak to

provide effective therapeutic IDUA activity in the brain. These findings represent the current conundrum facing gene therapy in the clinic in which only a relatively few cells take up therapeutic vectors for sustained expression that must suffice for all the other cells in the body, on both sides of the BBB. Strong expression can lead to activation of undesirable genes but weak expression is not therapeutically relevant. This may change when precise placement of therapeutic genes in targeted cells is achieved [129].

4. Outstanding Questions

4.1. Are mice good models for correction of MPS and LSD?

While mouse data are indispensible for proof-of-principle studies, they are insufficient in translation of protocols for clinical trials due to differences in anatomy, disease manifestation, sites of pathology, host responses to therapy, behavioral differences, and especially their totally inbred nature that reflects homozygosity at every genetic locus that researchers desiring experimental reproducibility employ in nearly every mouse-based study. As a consequence, most results have little potential for translation into the clinic [14, 130-133]. Moreover, there are issues of organ size, fundamental physiology and lifespan. The most common method of nonviral gene delivery for gene therapy in mice is hydrodynamic infusion. However, although several labs have tried to “scale up” the hydrodynamic delivery procedure to rabbits, dogs and pigs, no one has achieved effective delivery and sustained expression in larger animals [134]. After more than a decade of attempts by several laboratories, scaling up the procedure to animals weighing more than 2 kg has not been reported and it may not even be possible due to liver architecture in larger animals that precludes effective hydrodynamic delivery as commonly practiced [135]. Recent advances in gene editing now make it possible to create any genetic model in any animal, including those with physiology closer to humans [136].

4.2. How valid are current behavioral tests?

Neurologic and behavioral amelioration would be a definitive proof that gene therapy-mediated enzyme improves neurologic disease. However, in MPS it is often hard to pinpoint the site of amelioration that affects performance in a given behavioral test, since performance is a summary effect of correction of both the nervous system and somatic organs. Several studies have been dedicated to finding valid behavioral tests for mice, i.e., those that show significant differences between untreated MPS animals and their unaffected counterparts from the same breeding colony [109, 113, 137-142]. As is clear from **Tables II** and **IV,** comparison of behavioral correction across different studies is complicated because publications report results for only one or a few tests adapted for MPS I and IIIA. Therefore, functions evaluated in different studies may not be the same and tests with the same name may not examine the same parameters. Several behavioral tests used by some, do not show a difference between normal and affected mice in the experiments of others, as has been the case with a variant of the Morris Water Maze [113, 139, 140] and rotarod [113, 138] (and our unpublished observations). Some of the discrepancies may be due to a desire to present data that show positive therapeutic effects. Consequently, without full disclosure of all the behavioral tests performed, it is hard to compare functional effects of different treatment protocols.

4.3. What are appropriate biomarkers for CNS therapy?

Appropriate biomarkers are essential to assess quantitatively disease progression and therapeutic correction. Reduction of accumulated GAG is the most common biomarker used for assessing treatment of MPS disease. However, GAG measurements can be misleading because a high background of soluble GAG (or soluble proteoglycans) in the brain of WT mice can obscure MPS-related GAG storage [28]. In MPS IIIA, where GAG storage is in the form of heparan sulfate, quantification of total GAG by the dye-binding assay over-represents corrective effect in the brain, whereas the assay measuring HS *via* high-performance liquid chromatography is much more sensitive [120]. **Tables I** and **III** show that all the treatments considerably reduce or normalize total GAG levels even when full restoration of enzyme activity in the brain and neurologic correction are not achieved.

Blood analyses are commonly used to evaluate LSD progression. A readily available biomarker is the level of secondary elevation of other lysosomal enzymes, which rise in response to the primary enzyme deficiency and decline with restoration of activity. However, levels of secondary elevation of lysosomal enzymes in the serum are not indicative of changes in specific tissues. Molecular changes in other proteins in the blood may reflect pathological status in the CNS. Naughton et al. [143] performed a genome-wide gene expression array and showed many genes were significantly altered in both the brain and peripheral white blood cells of MPS IIIB mice. They were normalized upon restoration of deficient enzyme activity by an AAV9 vector, alongside near-complete correction of cytopathology. Genome-wide quantitative expression analysis in primary T lymphocytes performed by Cesani et al. [144] in a sample of patients with metachromatic leukodystrophy revealed specific metallothioneins whose overexpression correlated with disease progression and decreased with treatment. These examples demonstrate that the use of microarrays may assist in future evaluations of potential therapeutic protocols, particularly in combination with computer-assisted modeling [145].

4.4. Is insertional mutagenesis an issue for viral and non-viral gene therapy?

Insertional mutagenesis has always been an issue in gene therapy and has been thoroughly discussed from many angles. The plasticity and unexpected variability in human genomes as elucidated by recent whole-genome sequencing and the recently discovered high rates of remobilization of endogenous transposable elements suggest that integration by therapeutic vectors, including transposons, is not likely to induce adverse events when applied to MPS and LSD [36]. The use of LV-HSC that can be pre-scanned for growth characteristic before infusion into a patient further reduces the risk of adverse effects of insertional mutagenesis.

5. CONCLUSIONS

Our analysis shows that gene therapy for LSD involving systemic administration of either BBB-crossing AAV vectors (serotypes 9, rh.10, etc.) or LV-HSC is ready for clinical translation. For MPS IIIA disease, the first study in humans has successfully tested safety of AAVrh.10 by intracerebral injection via six burr holes [146]. AAV9-family vectors have a systemic effect via a single injection not only when administered into the blood circulation, but also into the CSF. Safety and efficacy of these treatments is currently evaluated in large

animal [77-79, 92]. The first LV-HSC clinical trial for metachromatic leukodystrophy in presymptomatic patients demonstrated that progression of the disease can be stalled and revealed no evidence of insertional mutagenesis or adverse effects of enzyme overexpression [32].

However, the detailed pioneering studies of Ponder, Haskins, Wilson and Hopwood in cat and dog models of MPS disease show that despite successful treatment of many somatic defects, not all are corrected [11, 14, 15, 73, 78]. In particular, neurobehavioral deficits are much harder to evaluate in larger animals whose "personalities" may vary and who are, in the words of Hemsley and Hopwood, "less amenable to statistically relevant, operator-blinded behavioral studies" [15]. For this, novel biomarkers and imaging technologies [147-149] are coming on line that may help quantify therapeutic correction in the brain. The prospects for successful DNA-based, liver-directed gene therapy can be significantly increased by improved "rationally designed" vectors [118]. The major challenge for non-viral vectors is their delivery to the liver in large animals. The success of the first human clinical trial for metachromatic leukodystrophy, a prototypical LSD [32] suggests that gene therapy for other MPS and LSD is right around the corner.

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Abbreviations

Apo	apolipoprotein
BBB	blood-brain barrier
BMT	bone marrow transplantation
CNS	central nervous system
CSF	cerebrospinal fluid
ERT	enzyme-replacement therapy
GAG	glycosaminoglycan
HSC	hematopoietic stem cell(s)
HSCT	hematopoietic stem cell transplantation
HS	heparan sulfate
IDS	iduronate sulfatase
IDUA	α-L-iduronidase
LDL	low-density lipoprotein
LSD	lysosomal storage disease
LV	lentiviral vector

PNS	peripheral nervous system
SGSH	sulfamidase
SP	signal peptide

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Highlights

- Gene therapy for LSD using LV-HSC and AAV9 or rh.10 vectors is ready for translation to the clinic
- Liver-directed gene therapy can ameliorate neurologic disease
- Efficiency of liver-directed gene therapy can be increased
- The major challenge for non-viral vectors is their delivery to large animals
- Evaluation of neurologic correction in animal models is inadequate

Table I

Enzymatic and metabolic effects of sustained IDUA activity in MPS I mice after systemic gene therapy

Target	Vector/Promoter/Dose[a]	Age Treated/Observed	IDUA activity (of WT)		Brain GAG or reduction in Storage vacuoles (SV)[c]
			Blood	Brain[b]	
NON-VIRAL APPROACHES					
		Unmodified IDUA [54, 67]			
Liver	SB(CAGGS) @1mg/kg	6-12 wk/4.5 mo	F: 70X, M: ≥100X	F: ≤6%(WB) M: n/a	Reduced 34%
Liver	MC(LSP) @0.5mg/kg	6mo/~3 mo	F:~100X	20-30%(Cb)	Reduced to normal
		Fusion IDUA-ApoE [121]			
Liver	Plasmid(LSP) @20g/kg	7-8wk/2 days	%WT n/a *60-120X*[d]	≤3%(WB)	Reduced to normal
VIRAL APPROACHES					
		Unmodified IDUA [75, 113]			
Liver	RV(LSP-IDUA) @1.7×10^{10}TU/kg	6wk/6.5mo	84X	73%(Fb)	Cb:Purkinje SV reduced 93% Cortex, Hc, middle & inner ear: SV reduced
	SIN-RV(LSP) @1×10^{10}TU/kg		25X	20%(Fb)	Cb:Purkinje SV reduced 65% Cortex, Hc: SV reduced
LV-HSC APPROACHES					
		Unmodified IDUA [115]			
Blood	LV-HSC(PGK) @10^6 cells	8wk/6 mo	100-220X	200-450%(WB)	Cortex: SV reduced; Cb: Purkinje density ≈ WT
		Fusion IDUA-ApoE [121]			
Blood	LV-HSC (ankyrin-1) @2-3 LV genomes/cell	7-8wk/5 mo	*12-20X*[d]	2-3 %(WB)	GAG ≈ WT

[a] TU, transducing units; the human (h) or canine (c) IDUA expression cassettes have either of the following promoters: ubiquitous (CAGGS, PGK), or LSP (liver-specific ApoEHCRhAAT) and erythroid-specific ankyrin-1

[b] n/a: not available; WB, whole brain; Cb, cerebellum; Fb, forebrain

[c] Hc, hippocampus

[d] relative to heterozygote values

Table II

Correction of behavioral, hearing and vision abnormalities in MPS I mice

RV-hIDUA-treated mice [75, 113]			
Test/parameter	**Improvement[a]**		**Function**
	IDUA activity in Forebrain (% WT)		
	23%	**73%**	
Sensorimotor tests			
1) Holding onto inverted screen	–	+	Balance, strength, motor coordination; vestibular function
2) Pole gymnastics	–	+	
Open-field tests			
1) Walking in first 5 min	+	+	Spontaneous locomotion
2) Whole-body movements	±	+	Environmental habituation
3) Rearings (vertical activity)	–	–	
Auditory test			
Auditory-evoked brain-stem responses	–	±	Hearing
Vision test			
Electroretinogram	–	+	Rod function

LV-transduced HSC-treated mice [115]		
Test/parameter	**IDUA in whole brain 2-5X WT**	**Function**
Open-field tests		
1) Rearings (vertical activity)	+	Spontaneous locomotion
2) Horizontal transitions	+	Environmental habituation
Auditory test		
Auditory-evoked brain-stem responses	±	Hearing

[a] improvement: +, complete or near-complete correction; –, no improvement; ±, partial but significant improvement

Table III

Enzymatic and metabolic effects of sustained SGSH activity in MPS IIIA mice

Target	Vector/Dos[a]	Age treated/tested	SGSH activity at termination (%WT)			Brain pathology	
			Liver	Serum	Brain	GAG or Storage vacuoles	Neuroinflammation
AAV APPROACH							
Unmodified mSGSH [79, 84, 96]							
Liver	AAV8 (LSP1) @10^{12}VG/mouse	8wk/10mo	F: 100% M: 400%	150% 450%	~4% 10%	F: No reduction M: 50% reduced	n/a[b]
Blood	AAV9(CAG) @7×10^{12}VG/mouse	2mo/10mo	F: 100% M: 500%	F: 100% M: 500%	6-8% 7-13%	GAG corrected SV reduced	near-resolved
CSF	AAV9 (CAG) @5×10^{10}VG/mouse	2mo/4mo	F: 20% M: 190%	20% 190%	7-39% 11-36%	corrected corrected except caudal part	resolved resolved
	AAV9 (CAG) @5×10^{9}VG/mouse					no reduction	n/a
Fusion IDSsp-hSGSH-ApoB [119]							
Liver	AAV2/8 (LSP2) @10^{11}VG/mouse	1mo/7mo	%WT n/a *700%*[c]	%WT n/a *200%*[c]	%WT n/a[b] *13%*[c]	near-corrected	resolved
LV- HSC APPROACH							
"Unmodified" cohSGSH [120]							
Blood	LV- (CD11b) @1.2-1.3VG/cell	2wk/8mo	60%	n/a[b]	11%	HS corrected	resolved in cortex and amygdala reduced in most regions

[a] VG, vector genomes; the murine (m) or human (h) SGSH expression cassettes have either of the following promoters: liver-specific LSP1= ApoEHCRhAAT; LSP2= thyroxine-binding globulin promoter; myeloid-specific = CD11b; co-codon-optimized hSGSH

[b] n/a, data not available

[c] relative to heterozygote values

Table IV

Correction of behavioral abnormalities in MPS IIIA mice

Test/parameter	Improvement[a]			
	Vector/route of delivery			
	AAV8 [96] i.v.	LV-HSC [120] i.v.	AAV2/8 [119] i.v.	AAV9 [79] intrathecal[b]
Accelerated rotarod				
1) latency to fall	–	n/d	n/d	n/d
Open-filed tests				
1) total distance travelled	n/d	+	+	+
2) number of lines crossed	n/d	n/d	n/d	+
3) number of rearings	n/d	n/d	n/d	+
4) frequency, duration of motion	n/d	+	+	n/d
5) time spent in central area	n/d	+	+	n/d
6) frequency of center entries	n/d	+	+	n/d

[a] Improvement: +, significant; –, no correction; i.v., intravenous; n/d, not determined

[b] high-dose AAV9@5×10^{10}VG/mouse