

Gene-modified neural progenitor cells for the treatment of neuropathic lysosomal storage diseases

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Lysosomal storage diseases: Lysosomal storage diseases (LSDs) are a family of about 70 disorders, with an overall incidence of 1:7000 live births. They are caused by dysfunctional lysosomal hydrolases, eventually leading to the accumulation of undegraded substrate into the lysosome. This results in a wide array of symptoms, which may include: the presence of dysmorphic features, cardio-respiratory disease, bone and joint disease, organomegaly, developmental delay and neurocognitive decline. The majority of these diseases have a neurological component and in the absence of treatment, death often occurs in the first decades of life, with the neurological complications drastically undermining the patient's quality of life, as well as their families (Boustany, 2013).

Current treatment options for LSDs: At present, the treatment of neuropathic LSDs is still a challenging area, due to the presence of the blood-brain barrier, which serves as a tight regulator of brain homeostasis. In this regard, enzyme replacement therapy, which consists of weekly or biweekly intravenous infusions of the recombinant enzyme, is only able to alleviate the somatic symptoms associated with LSDs. Treatment efficacy relies on a process termed cross-correction, namely the ability of the defective cells to take up the circulating enzyme via mannose-6-phosphate receptor-mediated endocytosis and restore lysosomal function. Hematopoietic stem cell transplantation also acts through the same cross-correction process, yet it provides a continuous source of the enzyme to the patient due to the ability of the monocytes derived from healthy transplanted hematopoietic stem cells (HSCs) to migrate, engraft into the recipient's brain and secrete the enzyme. Currently, hematopoietic stem cell transplantation is the standard medical care for severe mucopolysaccharidosis (MPS) type IH patients and it has proved to provide benefits to the central nervous system (CNS) impairment observed in MPSIH, and to a lesser extent, MPSII, and VII. Providing supraphysiological levels of the deficient enzyme by modifying the donor HSCs via HSC gene therapy (*ex vivo* HSCGT), broadens the applicability of this approach. Currently, metachromatic leukodystrophy is one of the best examples of HSCGT application for treating LSDs. A phase I/II clinical trial was conducted in metachromatic leukodystrophy children, who received autologous HSCs transduced with a lentiviral vector encoding the human arylsulfatase A (ARSA) cDNA, at pre-symptomatic or very early symptomatic stages (Sessa et al., 2016). Not only was ARSA activity restored both peripherally and in the cerebrospinal fluid, but most of the patients showed suspension of disease progression, a remarkable outcome in this disease. At the end of 2020, Libmeldy received EU marketing authorization for the treatment of metachromatic leukodystrophy. However, one patient who became symptomatic between apheresis and transplant was a poor responder on the trial, suggesting that treatment needs to occur in pre-symptomatic patients.

Cell death in LSDs is not addressed by current therapies: Overall, the current treatment options have the sole aim of providing the deficient enzyme, hence neglecting the need to replace any cells which are already extensively damaged. In Batten disease, there is evidence of compromised neurons and both inflammatory astrocytes and microglia seem to have a further detrimental effect on the surrounding cells, eventually leading to neuronal cell death. This is also the case in Niemann-Pick

disease type C, where the loss of cerebellar Purkinje cells, as well as reduction of glia in the corpus callosum, has been detected in the mouse model. In the case of Krabbe disease, the accumulation of psychosine, a highly cytotoxic sphingolipid, leads to oligodendrocyte death, demyelination, and multiple cellular dysfunctions. Since the progressive deterioration leads to a fatal outcome by 3 years of age, early intervention is imperative, but can only be truly effective if CNS regeneration is involved. This also concerns MPS diseases, where the appearance of abnormal neurological behaviors normally precedes obvious neuronal cell loss in animal models, thus suggesting neurological dysfunction at disease onset and subsequent progression of neurodegeneration (Bigger et al., 2018). In this regard, evidence for the need of early intervention has been provided by an intravenous AAV9 gene therapy clinical trial (NCT02716246), delivering the missing SGSH transgene, where improvement or stabilization of cognitive decline was reported to be achieved only in MPSIIIA patients treated prior to 30 months of age, as reviewed by Wood and Bigger (2022).

Developing novel regenerative therapeutic approaches: These studies are not alone in suggesting that earlier intervention in neurological diseases, especially those with primarily cognitive loss, might be critical for a positive clinical outcome in patients. Whilst lysosomal storage and dysfunction in cells can potentially be reversed by enzyme delivery, significant neuronal loss, as seen in MRI observation in patients with neuropathic lysosomal diseases, will not be solved purely by enzyme replacement. If we pursue a regenerative solution, to replace cells that have been lost, the primary challenges are to identify the important cells to replace, and in the case of neurons, to integrate these cells into existing networks. Whilst some of the lysosomal diseases – notably the leukodystrophies, may benefit from the replacement of oligodendrocytes producing myelin sheath material, almost all of the others would primarily benefit from neuronal replacement, which is what we focus on here. In 2007, Yamanaka and his group derived induced pluripotent stem cells (iPSCs), namely pluripotent stem cells generated from somatic cells, which can self-renew and differentiate into all cell types. Generally, delivery of iPSC-derived neural progenitor cells (NPCs) via intracranial injection seems to be an optimal choice to treat neuropathic LSDs, as once engrafted, these cells can differentiate into neurons, as well as potentially oligodendrocytes and astrocytes. Furthermore, CNS disease correction can be further enhanced by genetic modification of the NPCs to over-express the missing enzyme, prior to delivery. Indeed, in lysosomal diseases, overexpression of missing enzymes appears to be critical for success, probably because only a subset of cells in the body is ever targeted. What is interesting is how few neural stem cell therapies there are in the clinic, and this is for a number of reasons, including increased regulatory challenges if an advanced therapy medicinal product is classed as a tissue-engineered product rather than a cellular product. Another reason is that many groups are providing preclinical proof of concept, but often without addressing the International Society for Stem Cell Research (ISSCR) guidelines on stem cell research and clinical translation (ISSCR, 2021). In order to successfully translate this approach to the clinics, to improve regulatory confidence and to avoid undesirable therapeutic outcomes (Figure 1), we believe that these guidelines should be followed thoroughly.

Firstly, the cells should be extensively characterized to assess potential toxicities through *in vitro* and *in vivo* studies. In particular, risks for tumorigenicity must be rigorously assessed for any stem cell-based product, especially when derived from a pluripotent source. This is usually achieved by long-term studies assessing the human stem cells in xenogeneic models, for which immunocompromised rodents are often the preferable choice. Nevertheless, *in vitro* studies involving proliferation rate analysis and evaluation of oncogene or loss of tumor suppressor gene activity are also valuable. Furthermore, biodistribution studies should also be performed, to assess cell distribution throughout the body, expansion and differentiation. These should include delivery of the cell product using the intended clinical route and site of delivery and ideally should be performed in a good laboratory practice-certified animal facility. In this piece, we aimed to cover LSD research in the clinical application of iPSC-derivates and how they fit with ISSCR guidelines, but additional information can be found on the ISSCR official website.

A recent preclinical long-term study on Parkinson's disease represents the gold standard for stem cell therapy (Kikuchi et al., 2017), since all of the ISSCR guidelines were followed. Briefly, human iPSC-derived dopaminergic progenitors were characterized for all of the above aspects both *in vitro* and *in vivo* via transplant into the putamen of a primate model of Parkinson's disease. Following treatment, not only was no tumorigenicity observed for at least two years, but the injected neurons attenuated the disease from a histological and behavioral point of view.

iPSC-derived NPCs to treat neuropathic LSDs: Non-human animal models: To date, iPSCs have been used to correct neuropathology in several mouse models of LSDs. Proof-of-principle for the iPSCs potential to treat LSDs was established by transplanting murine iPSC-derived NPCs into the cerebral ventricles of neonatal MPSVII mice; following engraftment in the brain, the healthy donor cells led to widespread correction of lysosomal storage in both neurons and glia (Snyder et al., 1995). Regardless of these positive outcomes, the enzymatic expression provided by a healthy donor is not sufficient to reverse the advanced neuropathology observed in LSDs, for which supraphysiological levels of the deficient enzyme are usually required. In this regard, a step forward in the field was achieved by the combination of iPSCs with *ex vivo* gene therapy. In a Niemann-Pick disease study, murine NPCs derived from adult mouse brains were genetically modified to express human ASM and were injected into several brain regions of the ASM knock-out mouse model of type A Niemann-Pick disease. Transplanted NPCs engrafted and migrated towards several regions of the brain, leading to reversed lysosomal pathology, as well as regional clearance of storage material (Shihabuddin et al., 2004).

Human stem cells in xenogeneic models: Despite being an initial proof of concept, murine iPSC-NPC studies are not thorough for evaluating treatment efficacy. This is not only due to potential differences in differentiation protocols and outcomes, but also to the fact that non-human animal models may not replicate the full range of human toxicities associated with stem cell-based interventions. In this regard, human NSCs were genetically modified to express supraphysiological levels of β -galactocerebrosidase and subsequently injected into the cerebral ventricles of neonatal Twitcher mice, a mouse model of Krabbe disease (Neri et al., 2011). The transduced human NSCs were able to engraft and secrete β -galactocerebrosidase protein, leading to partial restoration of enzyme activity in the telencephalon and spinal cord. Nonetheless, as previously mentioned, to investigate the feasibility and safety of treatment at a pre-clinical level, immune-deficient rodents are required. In a more recent study on MPSVII, human MPSVII NSCs were genetically corrected through a transposon vector and then transplanted into adult NOD/SCID MPSVII mice. Following intraventricular transplantation, the NSCs engrafted into the CNS, yet correction of neuropathology was mainly restricted to the areas surrounding the injection tract (Griffin et al., 2015). Overall, there is supporting evidence of hampered migration of cells following intracranial injection

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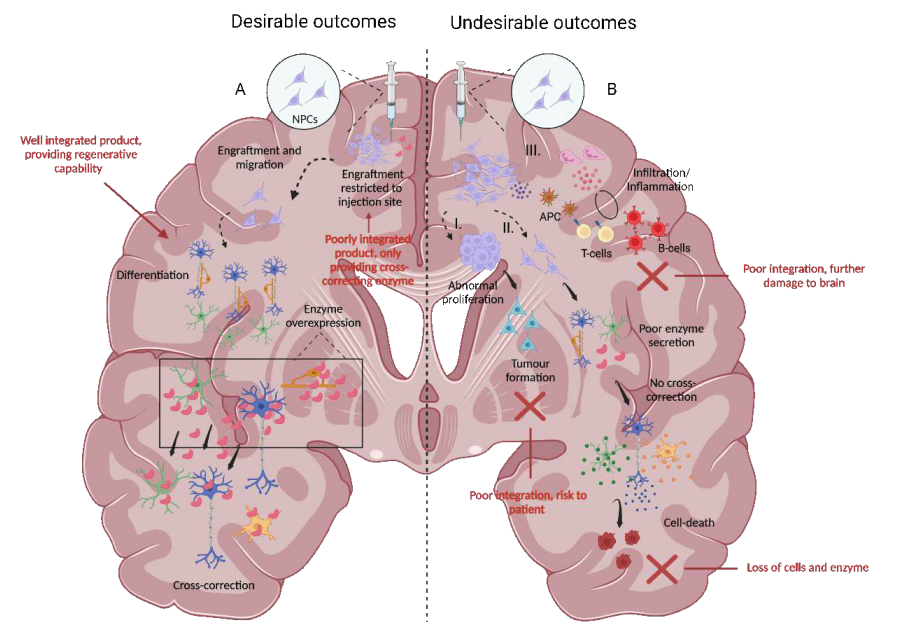


Figure 1 | Desirable and undesirable outcomes for cell delivery in lysosomal storage diseases.

(A) Typical outcomes of successful cell delivery: Following intracranial injection, the gene-modified NPCs engraft into the central nervous system and migrate from the injection site. They then differentiate into different cell types, which over-express the enzyme and are able to cross-correct the neighboring cells, eventually reversing neuropathology. (B) Typical outcomes of unsuccessful cell delivery: Following intracranial injection, partially differentiated NPCs can display abnormal proliferation, leading to tumor formation and metastasis (I). Alternatively, non-gene-modified NPCs can migrate and differentiate, but their poor enzyme secretion cannot cross-correct the damaged cells, which eventually undergo apoptosis (II). Lastly, the NPCs can be rejected upon engraftment by activation of the adaptive immune system, following antigen-presenting cells activation. T-cells migrate into the graft where they activate macrophages and granulocytes that have infiltrated in response to inflammatory stimuli; these cells, in turn, also help boost the adaptive immune response through the release of pro-inflammatory cytokines and chemokines (III). APC: Antigen-presenting cell; NPC: neural progenitor cell. Created with BioRender.com.

in MPS diseases. One potential reason behind this poor migration might rely on the relentless accumulation and high sulphation (N-, 6-O-, and 2-O) of heparan sulphate (HS) oligosaccharides in MPS diseases; in fact, it was proven that excess 2-O sulphated HS is able to bind and sequester CXCL12, eventually hampering hematopoietic migration in MPSI disease (Watson et al., 2014). Others have shown that fibroblast growth factor (FGF)-mediated signal transduction is only possible when at least one 6-O sulphate group is present on HS, thus implying a 6-O-sulphation-mediated FGF2/HS/FGF-receptor ternary complex generation. Since FGF-2 stimulates neural progenitor cell proliferation and regulates neural cell differentiation, abnormalities in HS may result in FGF-2 dysfunction and possibly to impaired neurogenesis. In this regard, a study on MPSIIIA showed that disease iPSC-derived NPCs displayed reduced proliferation, along with defective neuronal formation, and suggested the binding of excess soluble HS to FGF2 as a potential causative mechanism (Lehmann et al., 2021). Another possible reason behind the restricted migration of cells could lie in the use of NOD/SCID mice or other partly immunodeficient mouse models as the recipient of human NPCs. In fact, the engraftment and survival of xenogeneic cells are threatened by the host immune response, which can recognize the xenograft as non-self and destroy it. In this respect, the use of a highly immunodeficient mouse model of disease, such as the NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice, might increase the chances of cell survival and migration through the brain. These mice combine the lack of T-cell and B-cell activity of the SCID mice and the reduced macrophage activity of the NOD mice with the absence of interleukin-2 receptor γ , which blocks natural killer cell differentiation, and thereby removes a major obstacle for the engraftment of primary human cells, allowing patient-derived xenografts, as well as adult stem cells and tissues. Recently, a new MPSI mouse model based on an NSG background was generated. This model proved to live longer than 1 year, to be tumor-free and to be suitable for human cell engraftment. Although NPCs could be used as an enzyme factory in the brain, integration of some sort is the ideal scenario. Recently, integration of human neurons in rats, providing functional support has

been shown (Rust et al., 2022). There are of course challenges with potential behavioral changes induced by the integration of neurons, but these may be significantly less problematic than the course of some of these diseases like Sanfilippo (MPSIII), with severe behavioral and cognitive disturbance from the age of 2 years onwards.

Conclusions: In all, we propose that an iPSC-based gene therapy approach should be developed to treat those neuropathic LSDs patients with a late diagnosis or for which no treatment is currently available. Firstly, human healthy donor iPSC should be thoroughly characterized and differentiated into NPCs through a robust protocol. Once having assessed lack of off-target cells, the NPCs should be genetically modified to express supraphysiological levels of the deficient enzyme. Subsequently, any potential toxicity, tumorigenicity and biodistribution of cells must be rigorously assessed through *in vitro* and *in vivo* studies. In particular, the latter should involve the use of NSG mouse models of disease, and the long-term effects should be evaluated. Finally, a pre-clinical evaluation should be conducted in larger animals, before developing phase I clinical trials in human patients. One final problem remains, and that is the choice of using autologous cells from a patient that are corrected and reintroduced or using a well-characterized allogeneic cell line from a healthy individual. In either case gene modification to overexpress a missing enzyme would be beneficial in lysosomal diseases. The autologous approach is time-consuming and potentially too costly to be a realistic treatment for patients, whilst the allogeneic approach, although perhaps the most realistic and scalable approach across multiple diseases, especially where regulatory requirements for identity and purity may preclude the timely application of the former approach, will require immunosuppression to allow suitable persistence and integration of cells. Regenerative therapies of this kind could be conducted in the context of combination therapies, especially targeted anti-inflammatories or enzyme-based approaches that target the brain to slow neuronal loss. We hope that this article will stimulate further interest and development in this neglected aspect of lysosomal disease therapy.