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Nanomedicines to treat rare neurological disorders: The case of Krabbe disease

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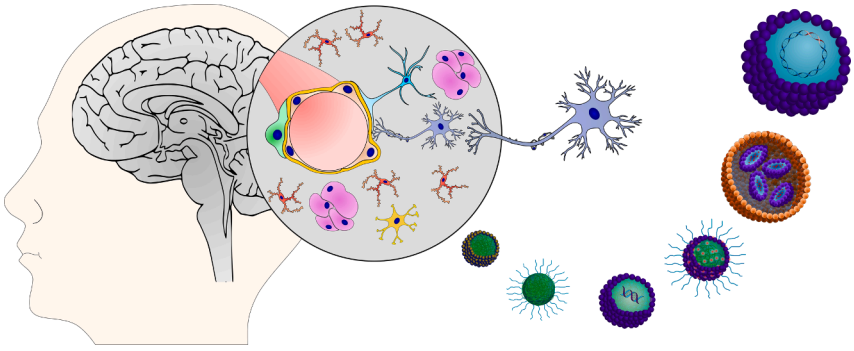
GRAPHICAL ABSTRACT



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ABSTRACT

The brain remains one of the most challenging therapeutic targets due to the low and selective permeability of the blood–brain barrier and complex architecture of the brain tissue. Nanomedicines, despite their relatively large size compared to small molecules and nucleic acids, are being heavily investigated as vehicles to delivery therapeutics into the brain. Here we elaborate on how nanomedicines may be used to treat rare neuro-developmental disorders, using Krabbe disease (globoid cell leukodystrophy) to frame the discussion. As a monogenetic disorder and lysosomal storage disease affecting the nervous system, the lessons learned from examining nanoparticle delivery to the brain in the context of Krabbe disease can have a broader impact on the treatment of various other neurodevelopmental and neurodegenerative disorders.

In this review, we introduce the epidemiology and genetic basis of Krabbe disease, discuss current in vitro and in vivo models of the disease, as well as current therapeutic approaches either approved or at different stage of clinical developments. We then elaborate on challenges in particle delivery to the brain, with a specific emphasis on methods to transport nanomedicines across the blood–brain barrier. We highlight nanoparticles for delivering

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therapeutics for the treatment of lysosomal storage diseases, classified by the therapeutic payload, including gene therapy, enzyme replacement therapy, and small molecule delivery. Finally, we provide some useful hints on the design of nanomedicines for the treatment of rare neurological disorders.

Nomenclature

AAV	Adeno-associated virus
ApoE	Apolipoprotein E
ASM	Acid sphingomyelinase
BBB	Blood–brain barrier
BPN	Brain penetrating nanoparticle
CED	Convection-enhanced delivery
CNS	Central nervous system
CXCR4	Chemokine receptor type 4
d_H	Hydrodynamic diameter
DNA	Deoxyribonucleic acid
DSPE	1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine
DTPA	Diethylenetriaminepentaacetic acid
ERT	Enzyme replacement therapy
FUS	Focused ultrasound
GALC	Galactocerebrosidase
GLD	Globoid cell leukodystrophy
HCT	Hematopoietic cell transplantation
ICAM-1	Intercellular adhesion molecule 1
iPSC	Human induced pluripotent stem cells
IV	Intravenous
% ID	Percent injected dose
KD	Krabbe disease
LDL	Low density lipoprotein
LNP	Lipid nanoparticle
LSD	Lysosomal storage disorder
LV	Lentiviral vector
mAb	Monoclonal antibody
MBP	Myelin basic protein
MOG	Myelin oligodendrocyte protein
MRI	Magnetic resonance imaging
MW	Molecular weight
PCL	Poly(caprolactone)
PEG	Poly(ethylene glycol)
PEI	Polyethyleneimine
PLGA	Poly(lactide-*co*-glycolide)
PVA	Poly(vinyl alcohol)
RVG	Rabies virus glycoprotein
SLN	Solid-lipid nanoparticle
SRT	Substrate reduction therapy
SPION	Superparamagnetic iron oxide nanoparticle
TfR	Transferrin receptor

1. Introduction

Krabbe disease (KD) is a rare monogenetic disorder that belongs to the class of lysosomal storage diseases. Untreated infants with early onset KD rarely survive past 2 years, and to date there is no cure. As a disorder primarily affecting the nervous system, researchers are looking for ways to combat KD's debilitating effects in an enduring and meaningful way.

Following the success of lipid nanoparticles as vaccines against SARS-Cov-2 [1,2], researchers are looking for ways to utilize nanomedicines against other pathological targets. One such direction is the engineering of nanoparticles to cross the blood–brain barrier (BBB) for the treatment of neurological diseases [3–9], and KD is one potential targeted condition for nanoparticle-mediated therapy. Here, we will highlight nanomedicines for the treatment of neurodevelopmental disorders, specifically using the lysosomal storage disorder KD as a framework to explore their potential. While KD is a specific monogenic disease, understanding how nanoparticles can be applied to treat this disease can provide a broader understanding for the treatment of other brain-specific disorders.

In this review, the pathology of KD will be elaborated upon, and its treatment will be framed in the context of nanomedicine. This will explore in vitro and in vivo models for preclinical studies of KD (i.e. the twitcher mouse model), while elaborating on clinical trials for treating KD. Furthermore, targeting and delivery methods for transporting nanomedicines across the blood–brain barrier in the context of KD will be discussed. Finally, examples of how nanoparticles can be used in gene therapy, enzyme replacement therapy, and small molecule delivery will be detailed, with specific consideration towards the treatment of KD and lysosomal storage disorders.

2. Krabbe disease

Krabbe disease (OMIM 245200), or globoid cell leukodystrophy (GLD), is an autosomal recessive disorder caused by mutations in the galactosyl ceramidase gene (*galc*) [10] resulting from a deficiency of galactocerebrosidase (GALC, EC 3.2.1.46), a lysosomal hydrolase. GALC deficiency results in the build-up of galactosyl ceramide and other undigested galactolipids, including psychosine (i.e. galactosyl sphingosine). Because oligodendrocytes and Schwann cells, i.e. myelinating cells, synthesize psychosine [11,12], their death due to psychosine cytotoxicity underpins KD-related demyelination [13–15].

Psychosine affects lipid rafts [16,17], increases membrane rigidity, and facilitates the microvesiculation and shedding of myelin [18], causing demyelination. The toxicity associated with the accumulation of psychosine is explained by two major hypotheses: i. psychosine exerts a non-specific "detergent-like" effect on the cell membrane [19]; ii. psychosine directly interacts with various proteins independent of their association with membranes to cause its effects [20]. The apoptosis of oligodendrocytes and oxidative stress induced by psychosine trigger multiple signaling pathways [21–23], including the stimulation of stress-activated protein kinases [24]. In addition, psychosine promotes the expression of cell death signals and simultaneously inhibits cell survival signals, such as the phosphoinositide 3-kinase [25–27]. Moreover, the inhibition of oligodendrocyte differentiation, widespread demyelination, and concomitant aberrant cell signaling have been suggested to be caused by apoptotic processes and abnormal inflammatory responses [28]. KD, in fact, is characterized by the presence of engorged multi-nucleated microglia called globoid cells [29,30]. The identification of globoid cells, often containing tubules in their cytoplasm, has been a defining feature of KD, although the specific function of these conspicuous cells has remained elusive.

2.1. Classification

Krabbe disease originally has been described as an infantile condition that was characterized by spasticity and a rapidly progressive neurologic degeneration leading to death [29]. More than 85 % of patients with KD have the infantile form of the disease, while the remaining 10–15 % of the patients have a later-onset form that can manifest itself in childhood or in adulthood. The major proportion of late-onset KD occurs between 3 and 10 years of age, but some patients have been healthy into their forties or even up to the age of 60. Infantile, juvenile, and adult forms of KD have a great variability in clinical manifestation [31]. Indeed, the clinical phenotypes of KD patients range from the classical infantile form, typically with an onset before 6 months of age and rapid progression, to late-onset forms (from 6 months to more than 9 years) with varying age of onset and rates of progression [32]. Although hundreds of genetic variants have been identified in the galc gene, definitive genotype-phenotype correlations remain elusive [33].

2.2. Epidemiology

The incidence of KD was originally estimated to be 1:100,000 [34]. Later, based on data from New York State Newborn Screening Program, Wasserstein et al. [35,36] reported an actual incidence of 1:394,000. A more accurate estimate of incidence in the United States was 1:250,000, as determined by analysis of death certificates [37]. However, KD was found with very high incidence (6/1,000 live births) in a large Druze kindred in Israel [38]. The incidence for KD is presumably that of the invariably fatal early infantile variant, most likely to be listed as a cause of death, and the deletion associated with early Krabbe is more present in Northern European countries [39]. The incidence of late-onset cases, which may have prolonged survival with more indolent symptoms, cannot yet be determined. Later onset cases appear to be more common in southern Europe, especially Italy and Sicily where the incidence of late-onset forms is likely underestimated. To date, no difference related to gender have been highlighted.

Diagnosis of KD is based on the demonstration of deficiency of the GALC enzyme and identification of the *galc* mutations. The GALC activity can be measured in leukocytes or cultured fibroblasts by radiolabeled natural substrate galactosyl ceramide or synthetic fluorescent substrate 6-hexadecanoylamino-4-methylumbelliferyl-D-galactopyrano side.

2.3. Genetics

In humans, the *galc* gene is located on chromosome 14 (14q31.3) [40] and has 17 exons [41]. The GALC protein comprises 669 amino acids and has six potential *N*-glycosylation sites that engage the mannose-6-phosphate receptor for trafficking to lysosomes [42]. In general, there is an inverse correlation between the amount of residual galactocerebrosidase activity and the clinical severity, but remarkable interfamilial variability of clinical manifestations has been described [43]. The disease is transmitted as an autosomal recessive trait. More than 200 *galc* mutations, including numerous small deletions, insertions, and numerous point mutations [31,44–46] have been reported in the Human Gene Database. Only a limited number of genotype-phenotype relationships have been established [47]. 86 infantile pathogenic variants have been identified; for these mutations, there is no report if they correlate specifically with the early-infantile or late-infantile phenotype [48–50].

2.4. In vitro models of Krabbe disease

GLD human cellular models include patient-specific fibroblasts [51,52], hematopoietic cells [53], or epithelial cell lines with induced galc mutations [54,55] that hardly recapitulate the metabolic and functional features of neural cells. However, experiments with primary cultures and cell lines of neurons, microglia, astrocytes, and oligodendrocytes have been used to study many processes such as neurotoxicity, inflammation, and neuroprotection and select new therapies for the treatment of neurodegenerative disorders, including KD. More recently, the development of new KD cell models has allowed the identification of neurologically relevant pathogenic cascades, including the major role of elevated psychosine levels. Based on these studies, the direct and indirect role of psychosine in triggering the release of cytokines, reactive oxygen species, nitric oxide, and in the activation of kinases, caspases, and angiogenic factors is becoming more clear [56].

The role of psychosine in forming globoid cells has been studied in vitro. In particular, microglia, but not macrophages, are activated and transformed into globoid cells using primary glial cultures in response to psychosine [57]. This transformation into globoid cells was found to be mediated by the extracellular protease matrix metalloproteinase-3. Claycomb et al. [58] extended these findings and determined that psychosine-activated microglia and globoid cells developed in this in vitro model system are toxic to oligodendrocytes and oligodendrocyte progenitor cells.

Likewise, psychosine has been added to neuronal, oligodendrocyte, Schwann, and/or fibroblast cell cultures to better characterize the role of inflammation in KD. Ribbens et al. [51] developed and characterized a new cell model for KD by obtaining brain samples from twitcher mice, the natural mouse model with GALC deficiency, and immortalized the primary neuroglial cultured cells with SV40 large T antigen, thereby generating the 145 M-Twi and the 145C-Wt cell lines from twitcher and control mice, respectively. Control and twitcher-derived cells were positive for markers indicative of oligodendrocytes, and the twitcher-derived neuroglialc ells showed elevated levels of psychosine. The twitcher-derived neuroglial cells futher showed decreased GALC activity and relative growth of the lysosomal compartment.

Human induced pluripotent stem cells (iPSCs) have been used to analyze disease pathogenesis in a patient-specific genetic background and test correction strategies. The differentiation of iPSCs in neural cells has boosted central nervous system (CNS) disease modeling and therapeutic screening [59]. Mangiameli et al. [60] established GLD patient-specific iPSC lines as a reliable human model to elucidate the pathogenesis of GLD and test the efficacy of gene therapy in relevant neural cell types. To this end, they differentiated GLD iPSCs into neural progenitor cells, differentiated progeny (oligodendrocytes, neurons, and astrocytes) and monitored the progression of cell-type- and patient-specific primary and secondary defects. They showed marked difference in the lipid profiles between GLD-patient derived cells and those from normal donors. They further assessed the impact of GALC reconstitution/overexpression (achieved by lentiviral-mediated gene transfer) in reverting the pathological phenotype and its potential effect on the biology of human neural progenitor cells and progeny, and in vitro gene therapy partially normalized the lipid profile of GLD-patient derived cells.

Thus, the use of in vitro testing has proven useful for elucidating the role of psychosine in KD-related toxicity, as well as study KD pathogensis (i.e. the formation of globoid cells from microglia and KD pathogenesis on a cellular level). While these in vitro cell cultures may provide insight into the pathogenic development of KD, they are scarcely able to evaluate the potential of therapeutic modalities. While initial screenings in vitro can test whether a therapeutic agent is able to reconstitute GALC activity or reduce substrate (i.e. galactosylceramidase) concentration, most investigations into therapy directly use well-known in vivo models because they are better able to recapitulate disease progression as well as the complex interactions between biological systems.

2.5. In vivo models of Krabbe disease

By 1990, it was known that KD was naturally occurring in five mammalian species including mice, cats, dogs, sheep, and rhesus monkeys. By 1997, the disease-causing mutations had been identified in

mice, dogs [63], and rhesus monkeys [64]. The spontaneously arising murine model of KD (twitcher) was first reported in 1980 [65]. Affected mice develop clinical symptoms at the onset of the active myelination period and, if untreated, die by about 35 days. Pathological differences, in comparison with wild-type mice, become evident around 15–20 days and twitcher mice become less active, fail to gain weight, and exhibit tremors. Terminal stage mice also exhibit paralysis, particularly of the hind-limbs and neck muscles, as well as a rapid loss of motor functions [66]. The pathology is very similar to that observed in human patients.

Phenotypic changes on a tissue-level can be seen in electron microscopy images of the sciatic nerves of twitcher and wild-type mice in Fig. 1**A-B** showing severe demyelination of axons [61], and histological images of KD-afflicted rhesus macaques' brain matter show characteristic large globoid cells in Fig. 1**C-D** [62]. Likewise, Wilson et al. [67] via electron microscopy and histology, specifically studying the peripheral nervous system (i.e. the sciatic nerve). Compared to wild-type mice, 35-day old twitcher mice showed more dispersed (less organized) nerve fibers with large numbers of mononuclear cells and higher levels of endoneurial connective tissue. Ultrastructural observations with electron microscopy showed excess Schwann cell processes forming around axons and deposition of collagen between those processes. At 25-days, macrophages were observed in the interstitial space and around nerves, and contributed to the breakdown of nerve architecture. Terminal stage (35-day old) twitcher mice on average had sciatic nerve cross-sectional areas 2-fold greater than wild-type mice. Immunofluorescent staining showed an increase over time of $CD68^{+}$ macrophages present in twitcher mouse sciatic nerve cross-sections, as well as significantly slower and generally lower myelin development compared to wild-type mice from 21 days after birth onward. These data show the dramatic neuroinflammation in twitcher mice, as well as the significant deterioration and structural changes to the nervous system as the disease progresses. Understanding the cellular mechanisms that trigger inflammation, the primary cells that initiate and respond to the inflammatory stimuli and identifying key immune signaling pathways involved in disease progression are critical areas for future research also important for the development of new therapies.

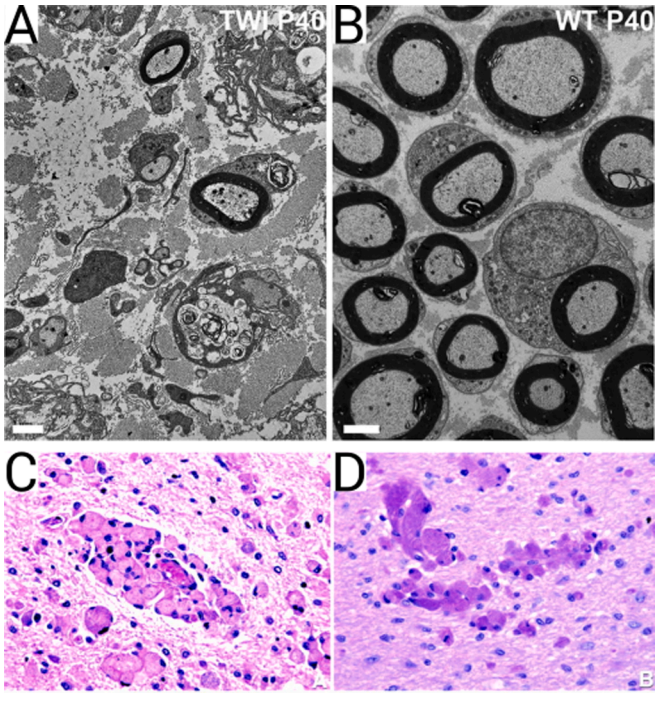

Fig. 1. Electron microscopy images from the sciatic nerves of (A) twitcher mice and (B) wild-type mice at 40 days after birth. Twitcher mice show marked demyelination and axonal damage. Adapted from [61], © 2018 with permission from Elsevier. (C) Hematoxylin and eosin and (D) periodic acid–Schiff staining histological images from the brains of rhesus macaques affected with Krabbe disease show the accumulation of large globoid cells in the brain. Adapted from [62], © 2008 with permission from Elsevier.

2.6. Therapy

Some therapeutic approaches have been attempted for KD: bone marrow transplantation [68,69], neural and mesenchymal stem cell transplantation [70–73], substrate reduction therapy [74], antioxidant therapy [23], pharmacological chaperone therapy [75], enzyme replacement therapy [76,77], gene therapy [78–82], and various combinations of these treatments [81,83–88]. Hawkins-Salsbury et al. [89], Reddy, and Sands 2011) and Nagabhushan Kalburgi et al. [90] have reviewed different therapeutic approaches for KD, and Table 1 shows ongoing or completed clinical trials for the treatment of Krabbe disease.

Hematopoietic cell transplantation (HCT) has been proven to be the sole effective therapy against KD, which generates cells that are thought to transfer GALC to myelinating cells. However, assessing the efficacy of cord blood transplantation is complicated by variable genotype–phenotype relationships in these patients. Furthermore, for effective treatment in children with the infantile-onset phenotype, transplantation must be performed before the onset of symptoms [93], but at this age HCT is associated with a 20 % mortality rate. Therefore, accurate diagnosis and prognosis are extremely important for the care of KD patients.

Substrate reduction therapy (SRT) is another approach that has shown promise in treating lysosomal storage disorders (LSDs), such as in type I Gaucher's disease [94,95]. By decreasing the synthesis of the primary enzyme substrate, SRT seeks to decrease the accumulation of pathogenic substrates and reduce lysosomal dysfunction due to the reduced pathogenic load. SRT has been explored in twitcher mice with the aim to slow the synthetic rate of the accumulating glycolipids. Substrate reduction therapy using L-cycloserine, an inhibitor of 3-ketodihydrosphingosine synthase, has been performed in mice. Litters were given subcutaneous injections of phosphate-buffered saline (PBS) or 75 mg/kg L-cycloserine. In twitcher mice, L-cycloserine treatment prolonged the lifespan by about 31 %, delayed the onset of clinical symptoms, and attenuated pathological signs [74].

Gene therapy, alone or in combination with stem cell transplantion, has been developed for almost two decades in mouse models, with increasing therapeutic benefit paralleling the improvement of next-generation adeno-associated virus (AAV) vectors. In vitro correction of the enzyme deficiency by retroviral vectors containing the *galc* cDNA was performed in fibroblasts, glial cells, astrocytes, and oligodendrocyte from twitcher mouse [96–99]. These studies demonstrated that oligodendrocytes from twitcher mouse can be biochemically and phenotypically corrected in vitro utilizing retrovirally mediated gene transfer as well as enzyme uptake. Specifically, the results of the experiments using AAV in the twitcher mice showed improvements such as prolonged life span, reduced psychosine levels, increased body weight and better performance in behavioral tests, but they die with symptoms similar to those of the untreated mice [78,79,82].

More recently, AAV2 genome construct expressing mouse GALC was packaged in AAVrh10 capsid: treated twitcher mice were active and symptom-free up to 8 months of age with a slower symptom progression compared to untreated mice [100]. Notably, it has been shown also that the combination of bone marrow transplantation with gene therapy prolongs life span even better than either treatment alone, indicating that replacement of GALC enzymatic activity is most effective when accompanied by modulation of immunity [81].

Lentiviral vectors (LV) was also applied to transfer a functional *galc* gene in the brain of twitcher newborn mice with a proficient transduction of proliferating and post-mitotic oligodendroglia [101]. This effort has recently shown remarkable efficacy in the canine model of the disease by one group that used either systemic or cerebrospinal fluid

Table 1
Clinical trial interventions for Krabbe disease[†].

Indication(s)	Treatment	Status	Clinical Trial Identifier/Ref.
Krabbe disease	FBX-101 single infusion (AAVrh10 carrying the GALC gene, following conventional hematopoietic stem cell transplantation)	Phase I/II (recruiting)	NCT04693598
Krabbe disease	PBKR03 injection (AAVhu68 carrying the GALC gene)	Phase I/II (recruiting)	NCT04771416
Inherited metabolic disorders	PBKR03 injection (AAVhu68 carrying the GALC gene)	Phase I (recruiting)	NCT02254863
Inherited metabolic disorders	Busulfan and fludarabine conditioning prior to hematopoietic stem cell transplantation	Phase II (recruiting)	NCT02171104
Inherited metabolic disorders	Hydroxyurea, Campath-1H, Fludarabine, Melphalan, Thiotepa with umbilical cord blood, matched unrelated donor bone marrow transplant, or peripheral blood stem cell transplant	Phase II (recruiting)	NCT01962415, [91]
Patients requiring stem cell transplantation	Human placental-derived stem cells with umbilical cord blood	Phase I (active)	NCT01586455
Inherited metabolic disorders	Enriched hematopoietic stem cell transplanatation	Phase I/II (active)	NCT01372228
Inherited metabolic disorders	Hydroxyurea, Campath-1H, Clofarabine, Cyclosporine A, Mycophenylate Mofetil, Melphalan, Antithymocyte Globulin and total body irradiation administered in days leading up to hematopoietic stem cell transplantation	Phase II, II/III (completed)	NCT00668564, NCT00383448, NCT00176904, [92]
Inherited metabolic disorders	Campath-1H, Busulfan, Cyclophosphamide, Cyclosporine A and Mycophenolate Mofetil administered in days leading up to hematopoietic stem cell transplantation	Phase II (completed)	NCT01043640
Inherited metabolic disorders	MGTA-456 ($CD34^+$ cell therapy) with hematopoietic stem cell transplantation	Phase II (completed)	NCT03406962

[†] Clinical trial search information comes from an https://clinicaltrials.gov search for "Krabbe disease" or "globoid cell leukodystrophy," Accessed: 26 Oct. 2022.

(CSF) administration of AAVrh10 or AAV9 [102]. These authors reported that a translationally feasible single administration of AAVhu68 expressing GALC into the CSF could mitigate most signs of Krabbe disease in the mouse and canine models, and paved the way for a first-in-human trial of AAVhu68.hGALC-administered intra-cisterna magna to infantile KD patients [102].

In vivo combination therapies have also been proposed relying on the effect of AAV-mediated, CNS-directed gene therapy, bone marrow transplantation, and SRT using L-cycloserine [87]. Not only did this triple combination increase the median life span of twitcher mice from ca. 35 days to ~ 300 days, but it also resulted in significant and persistent behavioral improvements. Preclinical experiments were conducted in a canine model of KD with a larger number of animals and different approaches [103]. The intravenous (at 3 days of age) and intracerebroventricular (at 6 weeks of age) injections of AAV of serotype rh10 (AAVrh10) to target, respectively, the peripheral and central nervous systems had no clear therapeutic outcomes. More recently, a study of intrathecal delivery of AAV9 showed a clear dose- and time-dependent effect in a canine KD model [104]. Here, AAVV9 encoding canine GALC was administered via a single intrathecal injection at high or low doses alongside an immunosuppressive dose of prednisone at either a presymptomatic (2 weeks) or symptomatic (6 weeks) stage. Remarkably, canines treated with a single high dose at the presymptomatic stage showed 100 % survival up to up to 16 weeks. Of these, six canines were held for long-term observation and were neurologically normal up to 1.5 years.

The majority of preclinical and clinical studies of in vivo gene therapy in lysosomal storage diseases are based on the use of AAV and LV, which are characterized by distinct cell/tissue tropism, particle distribution, persistence, immune issues, and oncogenic risks. The efficacy of LV (retroviral) delivery of GALC has been investigated not only in the twitcher mouse model but also in in rhesus macaques and patient iPSCs [60,101,105,106]. In a recent study, LV have shown the ability to reduce psychosine accumulation and partial rescue in terms of differentiation when tested on iPSCs [60]. Intracerebral injection of LV in twitcher mice showed effective production of GALC, not only in neurons but also in astrocyte and oligodenderocytes. However, improvement in either motor skills or life span was very limited [101,105]. The same results were observed when the study was conducted on rhesus macaques, although it was coupled with significant improvements in neuromuscular strength within 3 months post-therapy and scores were comparable to age-matched normal animals [106]. Nevertheless, considering the large size of LV (~100 nm) and their reduced ability to diffuse after inoculation, AAV (~20 nm) are preferred [107]. In particular, AAV have been used as vectors to restore GALC activity on Twi-trs, twitcher mice and a canine model of Krabbe disease. In any case, viral vectors hardly cross the BBB with the exception of AAV9 [108].

A cursory search of clinical trials for treating lysosomal storage diseases with AAV (i.e. condition or disease: "lysosomal storage disease" and other terms: "adeno-associated virus") reveals that most treatments are given intravenously (44 %), followed closely by direct intracranial/intracerebroventricular injection (40 %), 2/25 were intramuscular injections (2 %) and the final 2 % were follow up studies from previous treatments. AAV are thus highly pursued as gene delivery vehicles, but in order to improve brain-targeting specificity there is a tendency to inject directly into the brain, a more technically complex and invasive procedure [109,110]. AAV9 was found to exhibit a higher propensity for targeting the CNS, but like other viral vectors can be limited in efficiency and antagonized by native immunity. As such, a primary challenge in viral- or particle-mediated delivery remains overcoming the BBB.

3. Nanoparticles to target Krabbe disease

When considering nanomedicine for delivery to the brain, the BBB poses a formidable obstacle for the transport of macromolecules, drugs, nanoparticles, etc. into brain. The neurovascular unit (Fig. 2) is comprised of the basement membrane, specialized endothelial cells with tight junctions, and a host of supporting cells (e.g. pericytes, astrocytes, neurons, and microglial cells), that all act in concert to regulate brain homeostasis, limit the diffusion of small molecules and macromolecules, and mediate inflammatory response [111–113]. As such, the integrity of the BBB is of particular interest when considering neurological disorders [114].

However, it is further important to identify the pertinent physiological and cellular therapeutic targets and objectives for treating KD, i.e. to generate GALC in the CNS and peripheral nervous system and ameliorate neurodevelopmental/neurodegenerative effects due to GALC deficiency. Newly synthesized GALC is trafficked through the endoplasmic reticulum before passing through the *trans*-Golgi network and eventually into early/late endosomes then lysosomes [115]. Thus, the cellular targets of KD would be those where GALC deficiency leads to accumulation of psychosine (e.g. myelin-forming Schwann cells and oligodendrocytes), and as such delivering therapeutic nanoparticles across the BBB remains a formidable obstacle. Furthermore, one must

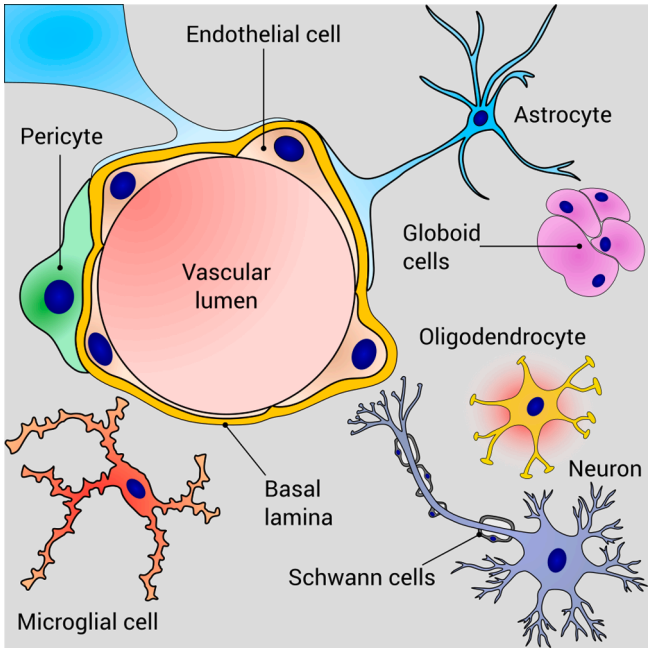

Fig. 2. Cross-sectional representation of the neurovascular unit (i.e. blood–brain barrier) showing the effects of Krabbe disease – The build up of psychosine leads to toxicity in oligodendrocytes and Schwann cells, leading to demyelination and axonal dysfunction.

consider KD-specific aberrations in the brain microvascular architecture and endothelium. Giacomini et al. [116] investigated changes to frontal cortex angioarchitecture of twitcher mice compared to wild type mice through immunoreactivity, microvascular corrosion casting followed by scanning electron microscopy, and quantitative RT-PCR. They found that twitcher mice had significantly less $CD31^+$, i.e. platelet endothelial cell adhesion molecule (PECAM-1) expressing, brain endothelium. Brain vasculature in twitcher mice further showed signs of prolonged neuroinflammation, and electron microscopy images of brain vasculature of twitcher mice showed dilated vessels and frequent changes in vessel diameter. Quantitative RT-PCR showed an upregulation in mRNA of *cxcr4*, *fgf2*, *cxcl-1*, *IL-1*, *tnf-α* and *CD45*. Immunofluorescent studies showed disorganization of perivascular pericytes and thus a reduction of endothelial coverage in twitcher mice, which indidcates changes in the BBB efficacy. Histological examinations by Li et al. [117] showed disorganized myelin and presence of globoid cells in the cerebellum of 36-day old twitcher mice, as well as the widesbread presence of $CD68^+$ (e.g. microglial) cells.

A review of endothelial cell dysfunction in Krabbe disease [118] shows that the cerebral microvasculature exhibits swelling of astrocytic end-feet around vessels, enlarged perivascular space, macrophage infiltration, dilated vascular lumen, and irregular shaped endothelium. A 1987 study by Kondo et al. [119] showed no increased permeability of the BBB to horseradish peroxidase in twitcher mice, but a histopathological study on samples derived from a 2.5-year-old patient with Krabbe disease showed marked defects in cerebral vascularization, such as decreased cortical microvascularization, irregular endothelium, and a decrease in smooth muscle coverage [120]. Thus, while it is not yet clear if Krabbe disease is associated with an increased permeability of the BBB, it is instead clear that neuroinflammation is a defining symptom of Krabbe disease [121]. The effects of neuroinflammation on the BBB permeability varies with the pathology, as it varies with the cause, location, and type of inflammation [122]. The exact cause of Krabbe disease-related neuroinflammation has not been elucidated but it is likely due to the cytotoxic buildup of lysosomal psychosine, and further work is needed to understand if nanoparticles can take advantage of this neuroinflammation in order to increase translocation across the BBB.

Taken together, nanoparticles may be able to exploit GALC-deficiency related alterations to BBB efficiency, as well as target receptors related to neuroinflammation or those found to be upregulated in the vasculature of KD. There are a number of strategies to facilitate nanoparticle delivery across the BBB, as highlighted in several excellent reviews [6,9,123,124]. Transporting nanoparticles across the BBB can be accomplished either by modulating particle properties (e.g. optimizing targeting ligand density/valency, coating particles with cell membranes, or by "super" PEGylation), through physical intervention (e.g. convection-enhanced delivery, hyperthermia, or focused ultrasound to transiently open the BBB) or by changing the route of administration (e.g. nasal delivery). Here, these approaches are surveyed in the context of neurodegenerative, and in particular lysosomal storage, disorders.

3.1. *Particle targeting approaches*

Movement of therapeutics across the BBB relies on active (e.g. receptor-mediated and adsorptive-mediated transcytosis) and/or passive (e.g. transcellular and paracellular diffusion) approaches. Researchers have found a number of targeting molecule candidates for localizing particles at or across the BBB [125–128]. Here, we review some of these strategies in the context of neurodevelopmental diseases (specifically lysosomal storage disorders like KD), where the BBB may be altered which can facilitate the delivery of nanoparticles into the brain.

Intercellular adhesion molecule 1 is a cell surface glycoprotein present on endothelial cells and regulates the extravasation of leukocytes at sites of inflammation [129]. Solomon et al. [130] systematically investigated the biodistribution following intravenous administration of nanoparticles targeted against different cell surface receptors or proteins, such as intercellular adhesion molecule 1 (ICAM-1), transferrin receptor (TfR), or monosialotetrahexosylganglioside, in healthy C57BL/6 or acid sphingomyelinase (ASM) knock-out mice. Niemann Pick disease is a lysosomal storage disease characterized by a deficiency in ASM, and is thus mimicked by the ASM knock-out mouse model. Following the injection of radiolabeled and targeted polystyrene nanoparticles (~200 nm), they could quantify accumulation in the brain through the localization ratio (i.e. the percent of injected dose/g, or % ID/g, in the brain relative to the % ID/g in the blood) as well as the specificity index (ratio of the localization ratio for targeted versus non-targeted nanoparticles in the brain versus liver). Anti-ICAM-1 targeted nanoparticles had significantly higher brain localization ratio in ASM knock-out mice (0.18) compared to control mice (0.09), as well as a significantly higher specificity index (6.4 versus 2.4, respectively). Immunohistochemistry imaging and Western blot analysis of ICAM-1 expression in tissues from lysosomal storage disease patients showed elevated levels of ICAM-1 compared to healthy (non-lysosomal storage disease) patients.

Transferrin is a critical glycoprotein responsible for the cellular transport of iron [131], and transferrin receptors are a class of transmembrane proteins expressed on endothelial cells of the BBB and responsible for the transcytotic delivery of transferrin into the brain parenchyma [132–134]. Clark and Davis [135] showed that 80 nm gold nanoparticles labeled with transferrin via an acid-cleavable linkage were able to cross out of the brain vasculature into the brain tissue. The acid-cleavable linker was crucial in this study because during the transcellular transport the gold nanoparticle core could be separated from the targeting molecule (and thus avoid receptor protein recycling/destruction). It was further shown that targeting ligand avidity was highly important. Johnsen et al. [136,137] likewise affirmed that targeting ligand affinity, valency, and density are critical factors for brain targeting. It was shown that a lower affinity was associated with a higher accumulation of gold nanoparticles in the whole brain homogenate, brain capillaries, and brain parenchyma [136].

The **insulin receptor**, another endogenous transporter which in part regulates the transport of glucose into cells, provides an avenue to target particles to the brain. It has previously been utilized for the delivery of

human serum albumin particles [138], solid-lipid nanoparticles [139], and gold nanoparticles [140] to the brain. Shilo et al. [140] decorated PEGylated 30 nm gold nanoparticles with insulin and followed the biodistribution in healthy BALB/c mice following tail vein injection. Using flame atomic absorption spectroscopy, they observed approximately 5 % ID reached the brain at 2-hours post-injection, a 10-fold increase compared to non-targeted PEGylated gold nanoparticles. However, it is worth noting that there was also a markedly higher distribution of particles to the liver and pancrease when nanoparticles were targeted with insulin. Even at 48-hours post-injection they detected 0.6 % ID of insulin-labeled gold nanoparticles in the brain, while the control PEGylated gold nanoparticles were not detected. In a follow up work, insulin receptor-targeted gold nanoparticles of varying sizes (20, 50, and 70 nm) were injected intravenously via the tail vein in male BALB/c mice [141]. It was shown that smaller insulin-targeted particles (20 nm) had the highest brain accumulation, and at two hours post-injection there was an approximately 2-fold increase in 20 nm gold nanoparticles in the brain (per gram tissue) compared to 50 nm gold nanoparticles.

Nicotinic acetylcholine receptors on brain microvascular endothelial cells and neurons specifically bind to the rabies virus glycoprotein (RVG), and are responsible for the neuro-invasivness of the rabies virus [142,143]. RVG has been shown to enhance retrograde axonal transport of the rabies virus once it has penetrated the CNS [144], and researchers have thus attempted to co-opt the functionality of RVG in order to target nanoparticles across the BBB [143,145–147]. Kim et al. [148] have employed RVG29, a 29 amino acid peptide derived from RVG, to target thermosensitive Pluronic-based nanoparticles (~60 nm) to the brain. Biodistribution of particles was monitored over 48 h using a fluorescent in vivo imaging system following intravenous tail vein administration in C3H/HeN athymic nude mice. Ex vivo imaging of brains showed that while both RVG- and RVG/chitosan-functionalized particles appeared to accumulate more in the brain compared to either bare or chitosan-only functionalized nanoparticles, the combination of RVG and chitosan accumulated significantly more. You et al. [143] showed that decorating the surface of poly(lactide-*co*-glycolide)-poly (ethlyene glycol) (PLGA-PEG) nanoparticles (~170 nm) with RVG29 significantly improved brain targeting when compared to untargeted PLGA-PEG, and in vivo experiments showed a 3-fold increase in brain targeting for RVG29-labeled particles 6 h after tail vein injection into C57BL/6 mice compared to non-targeted particles.

Low-density lipoprotein (LDL) receptors have been reported to be upregulated in brain endothelium [149,150], and this large endocytic recptor has been targeted as a potential pathway of therapeutics into the brain. LDL receptors regulate BBB permeability alongside tissue plasminogen activator, and are expressed in cells of the neurovascular unit [151]. Neves et al. [152–154] reported a series of studies investigating the potential of solid-lipid nanoparticles (SLNs) functionalized with apolipoprotein E (ApoE), a fat binding protein involved in lipid metabolism, to target LDL receptors for delivery to the brain. ApoE was conjugated to the SLN surfaces via two different particle components (DSPE-avidin or palmitate-avidin), both using biotin-avidin conjugation [152]. The resulting lipid nanoparticles were between 150 nm (bare SLNs) to 190 nm (targeted SLNs), as measured by dynamic light scattering. In permeability studies using hCMEC/D3 human brain microvascular endothelial cells and transwell cell culture devices, ApoE-labeled SLNs were shown to have a significantly higher apparent permeability (1.5-fold increase) compared to non-labeled SLNs. Subsequent studies showed that ApoE-labeled SLNs could increase the delivery of resveratrol, a natural polyphenol found in plants, across hCMEC/D3 monolayers [153]. Dal Magro et al. [155] showed that lipid nanoparticles (LNPs) with artificial ApoE adsorbed to the particle surface were able to increase brain targeting following intravenous injection. LNPs were incubated with recombinant human ApoE4 to form a so-called targeting corona, and ApoE-labeled LNPs showed a 3-fold increase in the brain after 30 min when compared to unlabeled LNPs.

Cell membranes have also been coated onto nanoparticles or used to form nanoparticles to achieve brain targeting [156], the rationale being that some peripheral cells in systemic circulation can naturally respond to cell signals on endothelium in order to leave circulation and go towards a target site. Dong et al. [157] were able to form monodisperse nanoparticles (190 nm) derived from “neutrophil” membranes (i.e. HL-60 human promyelocytic leukemia cells differentiated into neutrophil-like cells). These liposome-like nanovesicles were then loaded with Resolvin D2, a metabolite able to reduce leukocyte interaction with endothelial cells and reduce cytokine production. In a stroke model in male C57 mice, nanovesicles were able to successfully target the inflamed brain tissue as shown by ex vivo fluorescent imaging. This targeting effect was significantly greater compared to nanovesicles derived from non-differentiated cells as well as free fluorophore. Real-time fluorescent imaging of live mouse brain vasculature could also capture the neutrophil-derived nanovesicles localized in the brain capillaries. In a different study, ~150 nm PLGA nanoparticles were coated with cell membranes of neural stem cells that were first engineered to overexpress C-X-C chemokine receptor type 4 (CXCR4), a receptor for the lymphocytic chemotactic molecule stromal-derived-factor-1 [158]. Using a stroke model in male C57BL/6 mice, they showed that coating PLGA nanoparticles in the neural stem cell membrane enhanced particle localization at the stroke site, but this effect was magnified 2-fold when the neural stem cells were first engineered to overexpress CXCR4. These nanoparticles could be further loaded with glyburide, a diabetic medication that is also investigated for treatment of stroke, and particles targeted with CXCR4 were shown to dramatically increase mouse survival as well as the measured infarct volume from the stroke model when compared to both free glyburide and glyburide-loaded, membrane-coated nanoparticles (with no CXCR4 overexpression). These examples show that membrane-coated nanoparticles can increase targeting either by taking advantage of specific cell surface markers and natural cellular activity or by engineering cells to upregulate the expression of these markers.

3.2. *Non-receptor mediated approaches*

There are other interesting approaches to transport nanoparticles into the brain besides receptor-mediated targeting. This can include specific engineering of nanoparticle physico-chemical properties [159,160], or physically and transiently disrupting the BBB to facilitate particle transport [161,162]. A classical approach in nanomedicine to alter particle biodistribution, pharmacokinetics, and general interactions with the biological environment is to **specifically engineer particle physico-chemical properties.**

One such parameter is **nanoparticle shape**. Baghirov et al. [163] investigated the brain distribution of rod-shaped (300 nm long × 100 nm wide) mesoporous silica nanoparticles. It was shown that after intravenous tail vein injection in C57BL/6 mice, these particles were detected on the luminal side of the brain vasculature via two-photon microscopy. However, the rod-shaped nanoparticles were not found to cross into the brain parenchyma. This observation confirmed previous results by the group of Mitragotri [164], where it was shown that transferrin receptor-targeted, rod-shaped polystyrene nanoparticles (500 nm long × 120 nm wide) had a 7-fold increase in brain accumulation compared to similarly targeted spherical nanoparticles (200 nm diameter). However, it is important to note that in these works, the brain vascular endothelium was targeted, as the shape (and targeting molecule) enabled a preferential localization of rod-shaped particles in the brain vasculature relative to the spherical particles. The nanoparticles were in any case stymied by the BBB. However, these data emphasize an important piece of the puzzle in that particle shape can assist with brain localization.

Another important factor is the **particle surface** and **particle size**. Nanoparticle surface can be modified in a number of ways: changing functional groups to alter nanoparticle surface charge (i.e. zeta

potential), altering the particle surface chemical groups to change the constituent proteins of the so-called protein corona, or altering particle hydrophobicity/hydrophilicity. The group of Justin Hanes has systematically studied the effect of particle size, particle surface charge, and PEGylation density on nanoparticle diffusivity and penetration in brain parenchyma using multiple particle tracking in rodent and human brain tissue slices [165]. This would simulate the mobility of nanoparticles once in the brain. Polystyrene particles (40, 100, or 200 nm in diameter) were functionalized with either a negative (i.e. –COOH) surface or densely PEGylated. In ex vivo human brain slices, they showed that in all sizes the PEGylated particles had much higher mobility compared to the bare, negatively charged particles, as measured by their mean square displacement. Somewhat intuitively, it was also shown that smaller particles had higher mobility than larger particles. Further studies in ex vivo rat brain slices showed clearly that high density PEGylation was the most critical factor in ex vivo brain diffusivity, followed by size (i.e. $<$ 120 nm). These data were confirmed with in vivo experiments where nanoparticles were injected intracranially and particle diffusion was monitored in real-time through fluorescence microscopy.

Our own experiments on different sized nanoparticles confirms the apparent size threshold reported by Nance et al. [165] for enhancing brain biodistribution, specifically in twitcher mice. Fluorescently labeled silica nanoparticles (80 nm) and spherical polymeric nanoparticles (180 nm) were synthesized and characterized (Fig. 3**A-F**) before intravenously injecting in either healthy C57BL/6 mice or twitcher mice. Using a fluorescent in vivo imaging system to visualize explanted brains, it was shown that small silica nanoparticles accumulated significantly more in the brain in twitcher mice compared to healthy mice, and while the average fluorescent signal for the spherical polymeric nanoparticles was slightly higher in twitcher mice compared to healthy mice, these data were not significant (Fig. 3**G**). The rationale for these differences is obviously the size of the nanoparticles, but also that twitcher mice are characterized by severe neuroinflammation, which may compromise the BBB integrity. A recent work by Mahmoud et al. [166] investigated several of these aspects (i.e. size, shape, surface coating) with respect to the brain distribution of gold nanoparticles in Wistar rats, and PEGylated gold nanospheres were shown to accumulate in the brain at comparable levels compared to short PEGylated gold

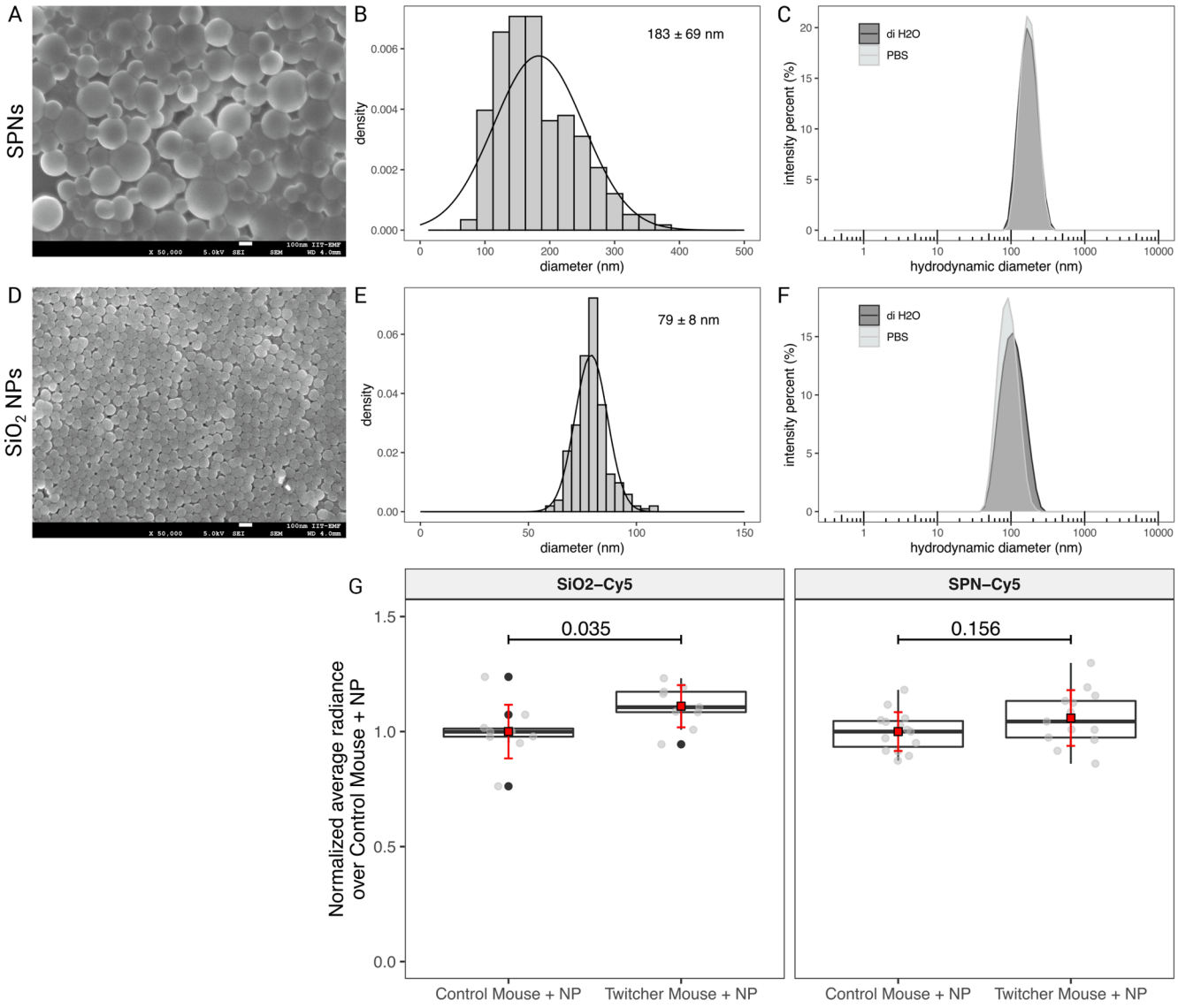

Fig. 3. Characterization of spherical polymeric nanoparticles (SPNs) and silica nanoparticles (SiO2NP). SPNs and SiO2NPs were characterized by (A,D) scanning electron microscopy (SEM), (B,E) histograms of particle diameters measured by SEM (n $\geq$ 230), and (C,F) hydrodynamic diameter was measured by dynamic light scattering. (G) Normalized fluorecent intensity of nanoparticle signal in different mouse brains. Values were calculated by normalizing radiant intensity by the average of control (i.e. healthy) mice with nanoparticles administered. Red squares with error bars indicate mean values $\pm$ one standard deviation. Black points indicate potential outliers (i.e. points greater than 1.5 $\times$ the interquartile range). p-values were calculated using a Student's *t*-test.

nanorods (3.3 versus 2.5 % ID, respectively) following intraperitoneal injection in male Wistar rats. The accumulation of both of these nanoparticles were significantly higher compared to long PEGylated gold nanorods (0.5 % ID).

Other methods have been explored to increase the transport of therapeutics (e.g. antibodies, small molecules, nanoparticles) into the brain parenchyma, such as by physically disrupting the BBB. One means to achieve this is through **magnetic resonance imaging (MRI)-guided focused ultrasound (FUS)**. In this approach, MRI is used to aid in the precise targeting of a specific location in the brain and FUS is applied to transiently disrupt the BBB. The use of FUS is generally accompanied by microbubbles which lower the acoustic pressure amplitude needed to transiently increase BBB permeability. When microbubbles and MRI-FUS are used in combination, detrimental effects such as thermal damage and microhemorrhaging are reduced [167]. The exciting results observed in the laboratory for MRI-FUS are supported by recent developments translating this procedure into the clinic [168], and cursory search of the U.S. Clinical Trials database (https://clinicaltrials.gov) indicates that there are around 70 active or completed clinical trials on transcranial focused ultrasound to treat pathological conditions ranging from tremors, brain tumors, Parkinson's disease, Alzheimer's disease, depression, amyotrophic lateral sclerosis, et al. MRI-FUS has been investigated for improving the delivery of nanoparticles into the brain for therapy or imaging. Nance et al. [169] showed that the delivery of 60 nm PLGA-PEG nanoparticles into the brain parenchyma was directly related to acoustic pressure. A follow-up work similarly investigated the combination of so-called brain penetrating nanoparticles, 50 nm self-assembled particles comprised of PEG-polyethyleneimine and plasmid DNA, with MRI-FUS and microbubbles showed that MRI-FUS improved nanoparticle accumulation in brain tumors by 2–3 fold compared to nanoparticles without MRI-FUS [161]. Moreover, efficacy of MRI-FUS was similarly dependent on acoustic pressure (Fig. 4), and confocal laser scanning microscopy showed that nanoparticles were broadly distributed out of the brain vasculature and into the tumor. A 2020 study by Ohta et al. [170] investigated the influence of size on nanoparticle transport across MRI-FUS permeabilized BBBs. Gold nanoparticles were administered intravenously in Institute of Cancer Research mice immediately followed by FUS administration at two different acoustic pressures. Intuitively, the accumulation of gold nanoparticles in the brain was directly related to acoustic pressure, and interestingly more 15 nm particles were retained in the brain (0.22 % ID at 0.7 MPa) compared to both the 3 nm (~ 0.06 % ID) and 120 nm (0.12 % ID) gold

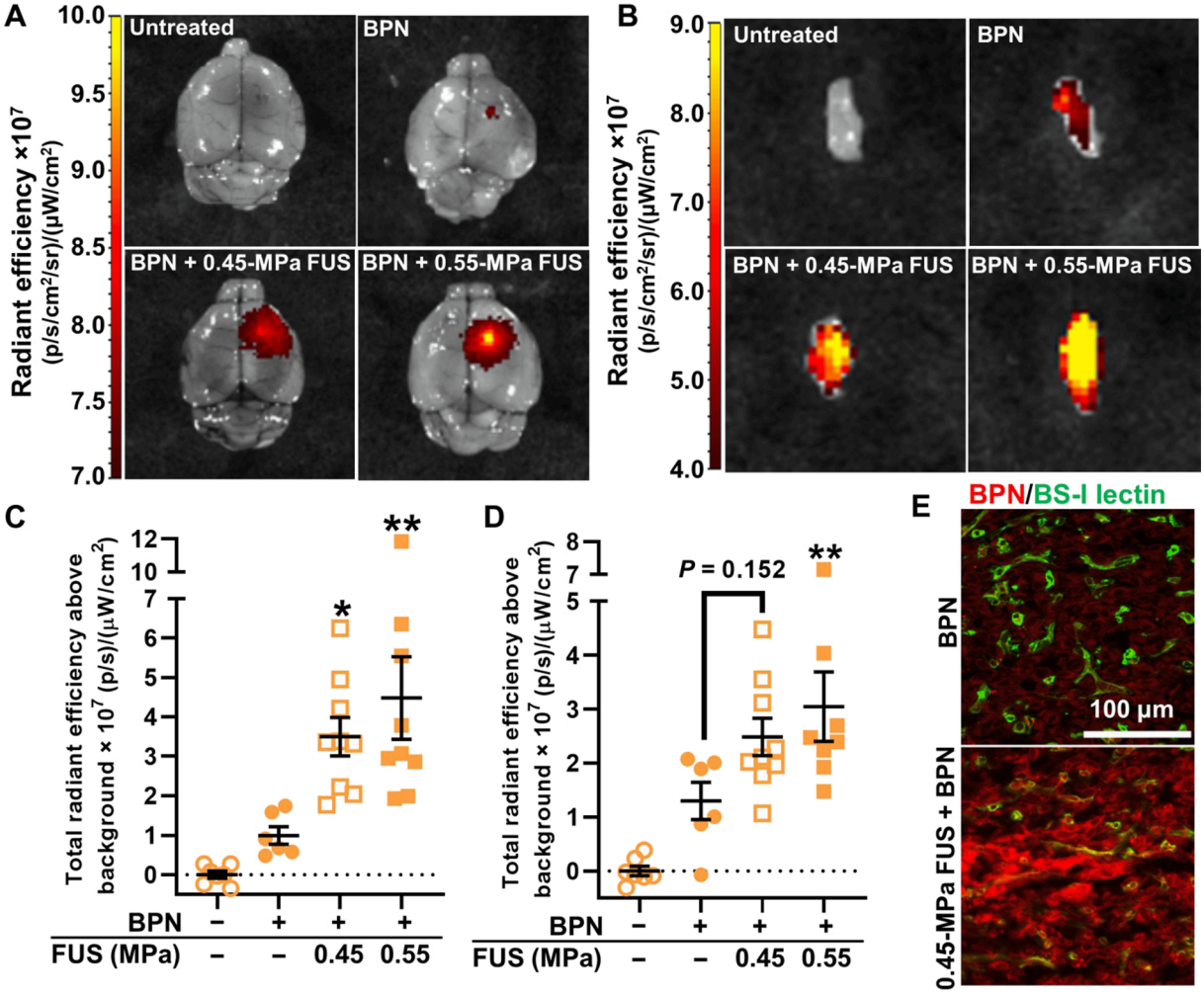

Fig. 4. Magnetic resonance imaging-guided focused ultrasound (MRI-FUS) improves the penetration of so-called fluorescent "brain penetrating nanoparticles" (BPNs) into U-87 glioblastoma xenografts following intravenous administration. Ex vivo imaging of (A) entire brains and (B) excised tumors qualitatively show how increasing acoustic pressure of the ultrasound, from 0.45 to 0.55 MPa, increases particle accumulation in the tumor. (C, D) Quantification of fluorescence signal intensity compared to control brains shows that MRI-FUS significantly increases accumulation of particles compared to BPN injected without MRI-FUS for whole brain images and excised tumors, respectively. (E) Confocal laser scanning microscopy shows the localization of BPNs (red) outside of brain tumor vasculature, marked by staining BS-I lectin (green). From [161].

nanoparticles. This phenomenon was attributed to the rapid clearance of such small particles from systemic circulation via the reticuloendothelial system (i.e. kidneys), and predictable size exclusionary effects for larger particles.

Hyperthermia, elevation of the body temperature, has been shown to increase the permeability of the BBB [171,172], and localized hyperthermia has been investigated as an approach to deliver therapeutics into the brain. For example, a 1977 study by Oscar and Hawkins [173] showed that exposing rats to 1.3 GHz microwaves under either continuous or pulsed exposure increased the permeability of different brain regions to different molecular weight (MW) radiolabeled molecules: [^{14}C] mannitol (MW = 182 Da), [^{14}C] insulin (MW = 5 kDa), and [^{14}C] dextran (MW = 60–75 kDa). Hyperthermia has also been applied to increase the delivery of nanoparticles across the BBB. Tabatabaei et al. [162] looked at the ability of magnetic iron oxide nanoparticles to permeabilize the BBB via radio frequency-induced hyperthermia. Magnetic particles were commercially available and were relatively polydisperse (ranging in diameter from 3 to 18 nm as measured by TEM) with a mean diameter around 12 nm. Using Evans Blue dye (as a model drug) alongside MRI imaging, they could confirm that magnetic heating could transiently open the BBB. These results were likewise shown in C57BL/6 mice administered with 5 nm magnetic nanoparticles and exposed to both an external magnetic field applied towards the brain and an alternating magnetic field (for nanoparticle heating) [174]. This combination showed a significant increase in iron content in the brain when compared to singular effects of free nanoparticles (i.e. no external magnetic field) or magnetic nanoparticles exposed to the external magnetic field without heating. Thus, elevating temperature, if done in a safe and controlled way, offers a means to transiently increase the permeability of the BBB and facilitate therapeutic delivery into the brain.

Convection-enhanced delivery (CED) is another means for improving the localization of therapeutics in the brain parenchyma. This is achieved by applying a continuous pressure gradient directly at the site of injection. This means there is a local injection directly in the brain, however the benefit is that this approach can push larger sized agents or hydrophobic compounds into the brain parenchyma where they will remain due to their limited diffusivity [175]. This approach has primarily been explored for the treatment of brain tumors, as these provide a localized intracranial physical target. For example, Xi et al. [176] investigated how using CED of doxorubicin coupled with nanodiamonds could increase brain retention of drug in healthy Fisher 344 rats. CED-administered nanodiamond-coupled doxorubicin (approximately 2–8 nm diameter [177,178]) was retained at the injection site significantly more than free doxorubicin up to 72 h after injection. Similarly, PLGA nanoparticles were injected by CED in the brains of healthy Sprague Dawley rats [179] and liposomes have also been used in conjunction with CED [180]. Liposomes loaded with temozolomide or Gd-DTPA as an MRI contrast agent were administered to rats via CED. Liposomes with Gd-DTPA showed enhanced MRI contrast up to 14 days after CED, while free Gd-DTPA was not apparent at the 2-day time point. Again, PEGylation played an important role in the retention of liposomes in the brain: PEGylated Gd-DTPA-loaded liposomes showed significantly higher distribution compared to non-PEGylated Gd-DTPA-loaded liposomes at 2 days post-CED. This approach is also a well-established, translational approach to increase particle delivery to the brain. A search of clinical trials for "convection-enhanced delivery" showed two studies using CED with liposomal irinotecan (NCT03086616, NCT02022644) for the treatment of brain cancers, with additional 4 × studies of CED for the treatment of Parkinson's and one for treating aromatic L-amino acid decarboxylase deficiency.

Intranasal delivery for direct nose-to-brain delivery has been proposed as a different administration route (e.g. compared to intravenous or localized injection) that can reduce operational invasiveness and, in turn, increase patient compliance [181–185]. This approach takes advantage of the direct connection between the olfactory region of the nasal cavity and the nervous system (Fig. 5). There are however unique challenges in this approach. Compared to systemic administration through intravenous injections, nanoparticles administered via the nasal route must overcome mucus and the nasal epithelium to gain access to either systemic circulation or the nervous system, and Mistry et al. [186] provide an in-depth review of the details of nanoparticle delivery to the brain via the nasal route. However, it is worth considering specific nanoparticle design characteristics to optimize nasal drug delivery. For example, it has been shown that, at the nanoscale, shape plays an important role towards the movement of nanoparticles through mucus. When comparing silica nanorods (80 nm × 240 nm, d_H 200 nm, aspect ratio = 3) against two different size silica nanospheres (80 nm or 140 nm core diameter, d_H 100 nm or 200 nm, respectively), it was shown that nanorods displayed significantly higher mobility compared to spherical particles in fresh mucus isolated from Sprague Dawley rat intestines [187]. Similarly, particle surface functionalization can greatly influence penetration through mucus. Yang et al. [188] showed that functionalizing polystyrene nanoparticles (~200 nm) with poly(vinyl alcohol) (PVA) at different percentages of surface coverage and different molecular weights could greatly influence the immobilization of particles in human cervicovaginal mucus (i.e. PVA acts as a mucoadhesive particle surface). Meanwhile, studies have shown that a PEG coating/increasing PEG coating density significantly increases particle mobility/diffusivity in mucus [189–191]. Thus, one must consider the trade-off between nanoparticle mobility through the nasal mucosa versus mucoadhesion, which could prolong the interaction time between nanoparticles and the mucosa of the olfactory region (i.e. increasing probability of particle uptake).

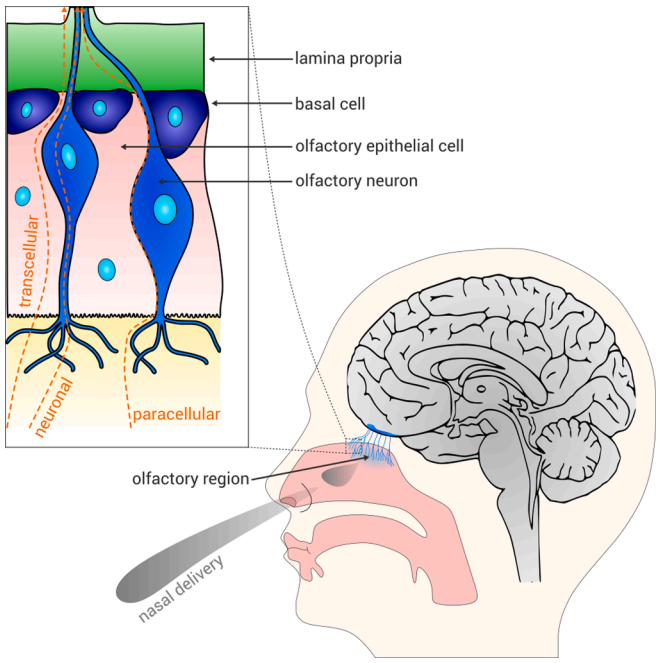

Fig. 5. Schematic showing the direct nose-to-brain route via the olfactory region of the nasal cavity. Transport of therapeutics can pass through the olfactory epithelium or directly into the olfactory bulb via intracellular axonal transport in the olfactory nerves.

3.3. Summary of brain targeting approaches for Krabbe disease

There is scant literature regarding the application of nanoparticles for the treatment of KD. However, the above mentioned approaches can provide insights into potentially successful avenues towards delivering therapeutic nanoparticles to the brain for gene therapy (i.e. DNA, RNA delivery), enzyme replacement therapy (e.g. delivery of GALC), or small molecule therapy to treat KD-related symptoms.

Active targeting approaches can exploit existing pathways present in cases of prolonged neuroinflammation (e.g. ICAM-1) or receptors shown to be upregulated in KD (e.g. CXCR-4). Likewise, it would unproductive to target receptors downregulated in KD endothelium (e.g. PECAM-1). Del Grosso et al. [192] investigated the delivery of PLGA nanoparticles carrying cross-linked enzyme aggregates of GALC to the brain of twitcher mice via peptides targeting angiopep-2, glyco-heptapeptide g7, and TfR. They found that, irrespective of targeting molecule, the targeted nanoparticles were better able to recover GALC activity in the brains of twitcher mice compared to non-targeted nanoparticles. This indicates that particle targeting is a critical component towards improving the therapeutic efficacy of particle-mediated treatment of KD. Further studies are required to elucidate if this is due to improving cell-specific delivery of GALC in the brain, if targeting molecules improve translocation of therapeutic particles into the brain parenchyma, or if targeting improves duration in which particles remain localized in the brain.

Further studies are also required to understand if non-active targeting approaches can improve the efficacy of particle-mediated therapy in KD. For example, Nance et al. [165] showed that densely PEGylated particles are better able to penetrate the brain parenchyma, while (smaller) size has also been shown to play an important role in particle localization in brains exhibiting neuroinflammation. It would be prudent to study whether approaches employing e.g. CED or MRI-FUS would better facilitate particle delivery in KD, especially considering developmental changes to the angioarchitecture and endothelium in KD.

4. Nanoparticles and therapeutic payloads to treat Krabbe disease

While the literature regarding the application of nanomedicines for the treatment of Krabbe disease is sparse, by looking at how nanomedicines are employed to treat other monogenetic neurological disorders it may be possible to understand how nanoparticles can be employed to help in the treatment of this disease. From a nanoparticle therapy standpoint, Krabbe disease can be approached from three different angles: i. gene therapy, that is to treat the genetic mutation in the GALC gene; ii. enzyme therapy, that is to replace the deficiency of GALC; iii. small molecule therapy, that is to treat and manage the symptom arising from this lysosomal storage disease. Table 2 highlights studies of nanoparticles for delivering to the brain the three different payloads, while Fig. 6 highlights the different types of particles and payloads.

4.1. Nanoparticles for gene delivery

Since its first discovery, gene therapy holds great promise for the treatment of so-called undruggable diseases. In recent years, this promise has started to be delivered thanks to the approval of several new therapies, marking the start of a “Golden Age” for the field. The approved medicines treat a wide range of clinical indications and tissue targets, including the first oligonucleotide-based therapies (Spinraza, Exondys, Vyondys), three cell therapies (Kymriah, Yescarta, Tescartus), and two in vivo gene therapies (Luxturna and Zolgensma), as well as the first RNA-based drug (i.e. Onpattro and the SARS-CoV-2 vaccines). On one hand these are life-changing for the affected patients, and on the other demonstrate a more general way forward by laying the foundations upon which treatments for many other conditions can be developed [193]. This is the case of KD, which being a recessive monogenic disorder, is an obvious candidate for gene therapy. In fact, despite the significant challenges, gene replacement, silencing, or editing are perhaps the most functionally straightforward options for the treatment of diseases caused by a single gene defect.

In general, there are two main approaches to affect the genetics of targeted cells: i. DNA-based therapeutics, which aim to provide a functional copy of a defective gene, or to cut DNA strands thereby stimulating DNA repair pathways to introduce desired sequence changes, or ii. RNA-based therapeutics, which allow the modification of gene expression without permanent changes to genome sequences [194,195]. Of note, the transient and reversible RNA-induced effect may potentially lead to greater efficiency and safety compared to the DNA-based technology [193].

Crossing the cell membrane and localizing into the appropriate subcellular compartment are well-known obstacles to the clinical translation of nucleic acid-based therapies, regardless of the specific differences in terms of chemical structure, target site, or mechanism of action of the nucleic acids used. This aspect, together with the very limited stability and need to limit side effects due to off-target action, makes the development of an appropriate carrier for the delivery of nucleic acid-based therapeutics pivotal. Thus, the common bottleneck in

Table 2
Examples in literature of brain-specific nanomedicines for treating neurodevelopmental/neurodegenerative diseases with gene therapy, enzyme replacement therapy, or small molecules.

Therapeutic class	Particle	Particle size (nm)	Targeting ligand	Therapeutic	Administration	Indication	Ref.
Gene therapy	AuNP@poly(N-(N-(2aminoethyl)-2-aminoethyl) aspartamide	~500	n/a	Donor DNA/Cas9	1 × intracranial injection	Fragile X syndrome	[199,200]
	PLGA-PEG	162	RVG29	Micro RNA-124 (miR-124)	Intraventricular IV (over 5-days)	Parkinson’s disease	[145]
	DNA nanoflower	~200	RVG29	Rutin/miR-124 chimera	6 × tail vein IV (every 5 days)	Alzheimer’s disease	[198]
	Liposome	~70	TfR mAb	pDNA	1 × jugular IV	Mucopolysaccharidosis, Type VII	[196,197]
Enzyme/ Proteins	PLGA nanoparticles	150–190	Ang2/g7/ Tf2	Galactosylceramidase CEA	1 × intraperitoneal injection	Krabbe disease	[192]
	PLA-PEG nanoparticles	~410	ApoE	β-galactosidase	(in vitro ony)	β-galactosidase-1 deficiency	[223]
	Liposomes	n/a	n/a	β-galactosidase	1 × intraperitoneal injection	Krabbe disease	[205,224]
	Liposomes	~100	TF2	Palmitoyl-protein thioesterase-1	(in vitro only)	Batten disease	[225]
Small molecules	poly(3-hydroxybutyrate-co-3-hydroxyvalerate)	~200–400	n/a	Fingolimod	(formulation only)	n/a	[210]
	PLGA	200–300	n/a	Fingolimod	1 × intrathecal or intralesional injection	Spinal cord injury	[209]
	SPION@PCL-PEG	140–240	n/a	Naproxen	1 × jugular IV	Magnetic targeting of brain	[226]

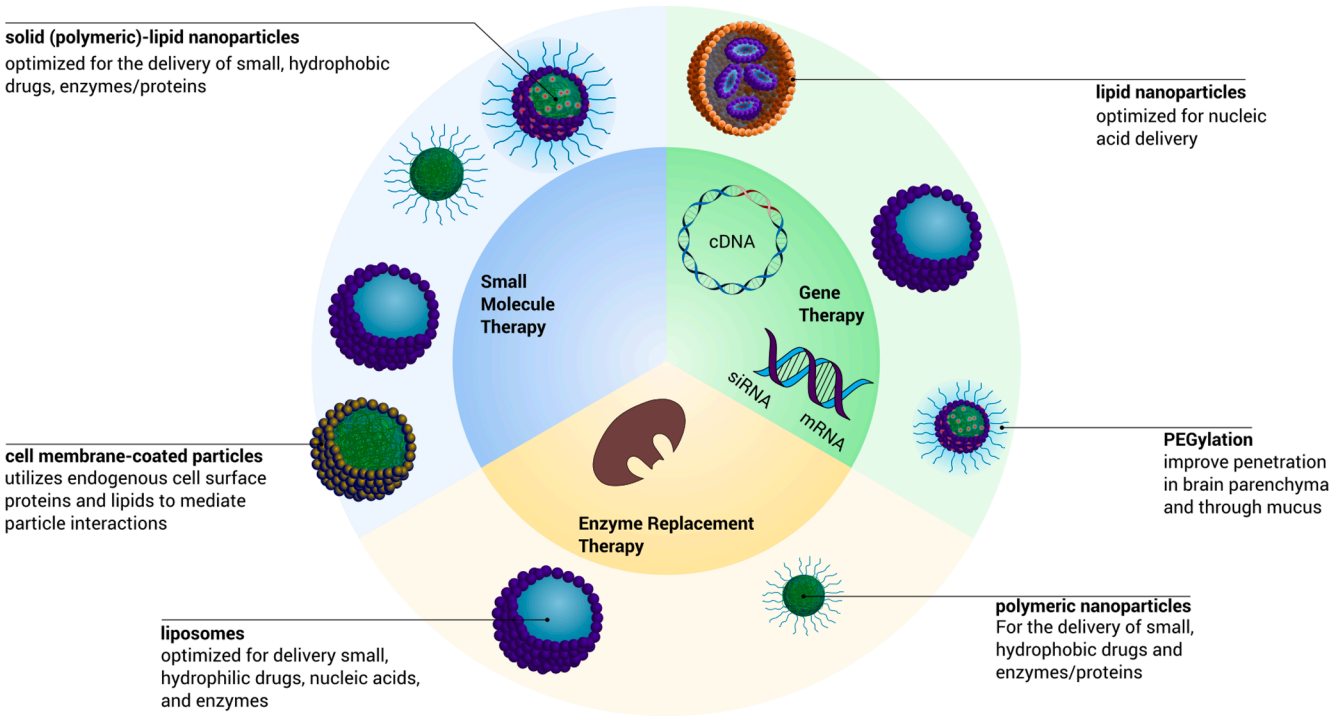

Fig. 6. Schematic showing different therapeutic approaches (i.e. gene therapy, enzyme replacement therapy, or small molecule delivery) as well as the different types of particles that are optimized for the specific therapeutic payload.

the translation to the clinic is the need for a carrier that could protect the genetic payload and deliver the nucleic acid at the target site.

It is well known that there is no one vector that is suited for all applications, but the gene transfer agent (i.e. nanoparticle) has to be carefully chosen depending on the cell type to be targeted, the number of treatments required (i.e. one dose versus repeated administration), and the size and nature of the nucleic acid to be delivered. While nonviral vectors are simpler and lack some risks inherent in viral systems, viruses are more frequently considered for gene therapy due to their innate adaptability and delivery efficiency. With their ability to "naturally" insert genetic material into host cells to replicate, viruses are efficient resources for gene therapy.

In looking at the literature, it is possible to find several examples of nanoparticle-based gene delivery approaches, specifically targeted towards the brain. Zhang et al. [196] developed liposomes encapsulating a plasmid for expressing β-glucuronidase (pCMV-GUSB), and targeting to the brain via monoclonal antibodies (mAb) for TfR. Previous studies reported that liposomes had a diameter of approximately 75 nm prior to functionalization with anti-TfR mAb [197]. Male MPS type VII GUSB null mice were administered pCMV-GUSB-loaded anti-TfR targeted liposomes, which increased serum GUSB enzyme activity by 5-fold, and therapeutic levels of GUSB activity in the brain were achieved. It is also possible to look at gene therapy approaches for other neurological disorders to identify how nanoparticles can be used in treating KD. Ouyang et al. [198] developed DNA "nano-flowers" (~200 nm) comprised of circular DNA that acts as a template for loading and delivering a micro RNA payload, specifically miRNA-124 for the treatment of Alzheimer's disease. Rutin was loaded as a small molecule with anti-inflammatory, anti-oxidant and Aβ inhibition, and RVG29 was included as a brain targeting motif. They showed the nano-flowers were able to significantly increase miRNA-124 targeting to the brain, however the particles mostly accumulated in the liver and kidneys. In a similar treatment paradigm, Gan et al. [145] loaded miRNA-124 into nanoparticles comprised of PLGA-PEG/PEI (~160 nm) for the treatment of Parkinson's disease. These particles could supress the mitogen activated protein kinase kinase kinase 3 levels in the substantia nigra of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-activated C57BL/6 mice, indicating an ability to inhibit a pro-inflammatory pathway.

In a different approach, Lee et al. [199,200] employed nanoparticles for the delivery of CRISPR-Cas9 for the treatment of fragile X syndrome. Particles were comprised of a gold nanoparticle core (15 nm) where a thiol-modified, single-stranded DNA was grafted to the particle surface. This grafted DNA had a complementary sequence to the donor DNA sequence, and the particle platform was further loaded with a guide RNA and the Cas9 protein. Lastly, the particle surface was functionalized with poly(*N*-(*N*-(2-aminoethyl)-2-aminoethyl) aspartamide) as an endosomal escape protein. This CRISPR-Cas9 delivery platform, called CRISPR-Gold, was injected locally in the brain and able to edit the mGluR5 gene, as well as rescue the exaggerated repetitive behavior in Fmr1 knockout mice, a murine model of fragile X syndrome [200]. These studies encapsulate some of the work going towards gene delivery with nanoparticles, however the scope has been specifically limited towards the brain.

4.2. *Nanoparticles for enzyme replacement therapy*

On a cellular/molecular level, Krabbe disease results in a deficiency in the lysosomal enzyme galactocerebrosidase. This has been conventionally treated via hemopoietic stem cell transplantation, and the majority of therapeutic clinical trials for Krabbe disease are targeted towards pharmaceutic regimes to improve donor engraftment, i.e. acceptance of the donor cells (Table 1). Thus, if a drug delivery vehicle was able to in some way consistently replenish this deficiency, one would expect to ameliorate the effects of this genetic disorder. A recent review excellently covers enzyme replacement therapy (ERT) for the treatment of lysosomal storage diseases, and discusses the application of nanomedicines in aiding this therapeutic paradigm [201]. In general, when rationally designing nanoparticle drug delivery systems one must consider the therapeutic payload as well as the target [202]. ERT, in the context of Krabbe disease and other lysosomal storage diseases, strives for the delivery of enzymes specifically to the lysosomal compartment. Conventional ERT can be challenging due to the difficulty of

systemically administered enzymes crossing the BBB, enzyme immunogenicity and adverse reactions, enzyme stability, and finally due to the transient effect of ERT as a treatment option. Nanoparticles may provide a means to overcome some of these limitations.

Del Grosso et al. [192] employed PLGA nanoparticles to deliver cross-linked enzyme aggregates of galactosylceramidase. PLGA nanoparticles (150–190 nm) were formulated which were targeted to the brain via different targeting peptides: angiopep-2, glycosylated heptapeptide g7, and transferrin binding peptide. They showed that the combined response of targeted nanoparticles recovered galactosylceramidase activity in twitcher mice brains (42 % of the enzyme activity measured in healthy wild type mouse brains), a level comparable to untreated heterozygous control mice (45 %) (Fig. 7). Meanwhile, the combined response of free galactosylceramidase or non-targeted galactosylceramidase-loaded nanoparticles administered to twitcher mouse had a markedly lower recovery of activity (10 %) compared to healthy wild type mice. These data show the importance of brain targeting towards the enzymatic treatment of lysosomal storage diseases, in this case specifically Krabbe disease.

PLGA nanoparticles have also been investigated for the delivery of fluorescently labeled albumin as a model high MW therapeutic (i.e. enzyme) [203]. Nanoparticles were loaded with fluorescent albumin and targeted towards the brain with the g7 peptide (250 nm), and then systemically administered in both Idua knock-out (mucopolysaccharidosis I) and IdS knock-out (mucopolysaccharidosis II) mice. Fluorescence imaging of brain sections showed that g7-targeted nanoparticles accumulated more in the brains of both Idua knock-out and wild type mice compared to untargeted particles, and g7-targeted particles

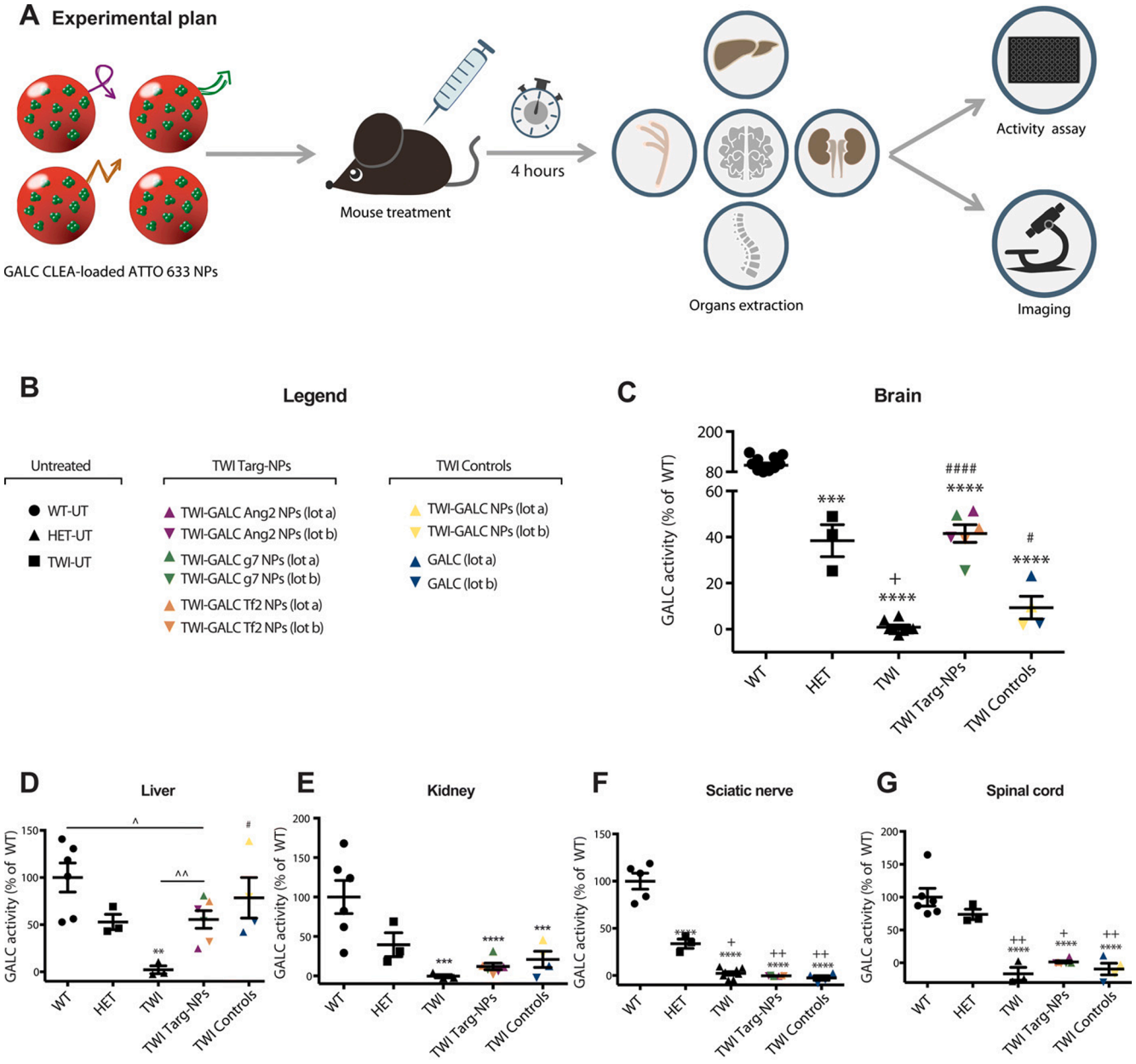

Fig. 7. Twitcher mice were administered PLGA nanoparticles loaded with cross-linked GALC aggregates and labelled with a targeting molecule. GALC activity was measured in various organs at 4 h after injection, and targeted particles were showed to recover GALC activity in the brains of twitcher mice up to 40% compared to wild type mice, levels that were equivalent to heterozygous, non-pathological mice. This was a significant improvement compared to both untargeted particles or free GALC. From [192].

accumulated more in the brains of Idua knock-out mice compared to wild type mice. Similarly, targeted particles accumulated more in the brains of IdS knock-out mice compared to wild type mice. These two studies emphasize the importance of targeting in brain delivery of enzymes. For example, PLGA nanoparticles with arylsulfatase B conjugated to the particle surface were unable to effectively increase enzyme activity in the brain due to poor brain biodistribution of particles [204]. Moreover, although not a direct comparison, it is useful to consider a 1985 study by Umezawa et al. [205] where β-galactosidase encapsulating liposomes were injected in twitcher mice for ERT. They were unable to show a significant effect of the exogenous enzyme in clearing accumulated lipids in twitcher mice brains. Even in 1985 the authors emphasize the challenge and necessity to find ways to deliver their liposomes across the BBB.

While there are relatively few manuscripts on nanoparticles for the treatment of Krabbe disease, by expanding the scope to consider nanoparticles for enzyme replacement therapy some general conclusions can be formed. First, the nanoparticles must be capable of load a relatively high MW payload, that is, enzymes. As discussed, this can either be recombinant human enzymes or cross-linked enzyme aggregates. Thus, in general enzyme delivery will be mediated through either polymeric nanoparticles or through liposomes. Secondly, in order to cross the BBB, nanoparticles either need to be modified with a targeting ligand or other steps need to be taken to ensure particle localization in the brain. Finally, as ERT is a transient solution, one must consider ways to optimize particle dosing and the delivery route.

4.3. Nanoparticles for small molecule delivery

There are no current small molecule interventions approved for treating Krabbe disease (Table 1), and any small molecules in clinical trials are generally included for improving stem cell transplantation. Thus, the outlook for delivering a therapeutic small molecule to manage Krabbe disease is suboptimal, and nanoparticle-mediated delivery of a drug would then be intended to manage the severe symptoms associated with Krabbe disease, such as demyelination [206] and chronic neuroinflammation [207].

Remyelination is a critical area of research for many neurodegenerative and neurodevelopmental diseases. Myelin, the insulating sheath that forms around axons, serves a critical function in both supporting axonal metabolism as well as facilitating nerve signaling by insulating signal transduction along the axonal pathway. Oligodendrocytes are the cells responsible for forming myelin, and are thus the cellular target of remyelination therapies. Small molecules have been shown to facilitate remyelination in vivo. Recently fingolimod, a drug active against the sphingosine 1-phosphate receptor, was shown to significantly rescue myelin levels in twitcher mice as well as decrease immobility, decrease twitching severity, and prolong survival time [208]. In this study, fingolimod was administered in the drinking water to a calculated final dose of 1 mg/kg/d. Fingolimod has also been encapsulated in PLGA nanoparticles (d_H 225 nm), and when delivered locally in conjunction with neural stem/progenitor cells were able to promote recovery of motor function following spinal cord injury [209]. Shahsavari et al. [210] also reported the use of a neural network to formulate fingolimod-loaded poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) nanoparticles.

In order to find molecules that could increase myelination, Najm et al. [211] screened a library of drugs for their capacity to enhance the generation of mature oligodendrocytes from oligodendrocyte progenitor cells. They identified two drugs, miconazole and clobetasol, which were able to show an increase in myelin basic protein (MBP) fibers in ex vivo cerebellar slices taken from day 7 postnatal mice (i.e. before significant myelination). Image analysis of immunofluorescent stained MBC and independent Western blot studies showed that these two drugs increased myelination, and these data were validated in vivo with a focal demyelination model in female C57BL/6 mice. Clobetasol and miconazole were able to increase the number of new $CC1^+$ oligodendrocytes in demyelinated lesions in spinal cord white matter, and also increase MBP staining in drug-treated lesions. Clemastine, an antihistamine with anticholinergic effects, is another drug that has shown potential for affecting remyelination [212,213], and currently there are several clinical trials investigating the remyelination potential of clemastine (NCT03109288, NCT05338450, NCT05359653, NCT02040298, NCT02521311, NCT05131828). Results of one of these clinical trials (NCT02040298) was detailed in a 2017 report by Green et al. [214] and showed that clemastine could decrease latency of pattern-reversal visual-evoked potentials in multiple sclerosis patients. Increased latency is a measure of multiple sclerosis-related demyelination of the optic nerve. There exists little literature on nanoparticle formulations with clemastine, as this pharmaceutic already used in the clinic is generally administered orally, however supercritical antisolvent precipitation has been used to formulate clemastine nanoparticle crystals [215]. It is conceivable to consider the loading of clemastine, generally sold as a fumarate salt, into various materials due to it's solubility in aqueous solutions as well as organic solvents (e.g. ethanol, dimethyl sulfoxide, dimethyl formamide).

Nanoparticles have been much more investigated for the management of neuroinflammation [216]. Clementino et al. [217] presented an interesting approach towards using nanoparticles to control neuroinflammation in the treatment of KD – That is, rather than deliver an active pharmaceutical agent, particles acted as a sponge to absorb the neurotoxic sphingolipid psychosine both in vitro and ex vivo in mouse cerebellar organotypic cultures. Lecithin/chitosan nanoparticles (234 nm) were able to recover both myelin oligodendrocyte protein (MOG) and MBP in cerebellar organotypic slice cultures from C57BL/6 mice. Brain slices insulted with psychosine showed a dramatic loss of both MOG and MBP, but when treated in conjunction with lecithin/chitosan nanoparticles the expression of these two proteins was recovered. Moreover, dynamic light scattering studies of nanoparticles in solution with psychosine showed an increase in size, hinting at the physical alterations to particle structure due to interaction with psychosine. These data were confirmed with cryo-transmission electron microscopy. However, a more conventional approach is to use small molecules to mitigate neuroinflammation. Luzi et al. [218] investigated three different anti-inflammatory drugs for their ability to treat a Twi-trs mouse model of KD. Mice treated with ibuprofen, indomethacin, and minocyclin showed improved survival times compared to untreated mice. Indomethacin has also been reported to enhance remyelination [219], and has previously been formulated into nanoparticles [220–222].

4.4. Challenges in nanoparticle therapy for Krabbe disease

The application of nanomedicine towards treating neurodevelopmental and neurodegenerative disorders has gained attention, and while not many studies have investigated KD as a therapeutic target, there is potential for nanomedicine to make a difference. As a recessive monogenic disorder, gene therapy is the most obvious therapeutic approach. However, nanoparticle-mediated gene therapy can be challenging due to limitations in manufacturing, scalability, and targeting. Furthermore, effects of non-viral gene therapy can be transient, thus necessitating repeated administrations. In the context of KD, this means repeated administrations that must overcome the BBB to reach the therapeutic target. Likewise, enzyme-based therapies would necessitate multiple administrations. Whereas gene therapy could potentially rectify the underlying basis of KD, i.e. the recessive mutation on the galc gene, ERT or SRT would function by supplementing GALC in the deficient tissues or reducing the psychosine precursors (i.e. substrate) in the tissue, respectively. Thus, ERT would rely on repeated administrations to maintain normal levels of GALC in tissues of the CNS and peripheral nervous system.

While gene therapy and ERT, in essence, aim to resolve the pathological basis of KD (i.e. a deficiency of GALC in the CNS and peripheral nervous system due to a recessive monogenic mutation), the goal of

small molecule therapy is to mitigate the effects of GALC deficiency, such as prolonged neuroinflammation and demyelination. While delivery of therapeutic small molecules does not resolve the underlying basis of KD, it could be that this approach using nanomedicines could treat neuroinflammation, help reverse demyelination, and assist or halt the development of disorganized angioarchitecture in the brain. There are several clinical trials investigating therapeutics such as clemastine for remyelination, however few have looked at using nanoparticles as a delivery vehicle. Other small molecules such as clobetasol or miconazole have been shown to increase proliferation of oligodencrocytes. Systematic studies are needed to show if packaging these therapeutics into a nanoparticle drug delivery system, perhaps in conjuction with a brain-specific targeting approach, could improve their utility in treating KD-related symptoms.

What becomes apparent is that a multi-pronged approach is necessary to treat KD, and nanomedicine may have a role to play. While viral-based therapies are having success in clinical trials, it may be that nanomedicine may assist – either through ERT or delivery of drugs that can repair some of the damage wrought by KD-related GALC deficiency.

5. Summary and conclusions

Krabbe disease is but one of any number of neurodegenerative or neurodevelopmental disorders. However, as a lysosomal storage disease, a monogenic disorder, and rare orphan disease, there are important benefits to exploring how nanomedicines can be applied towards its treatment. KD is in part characterized by chronic neuroinflammation, a pathology shared by many neurodevelopmental disorders. Moreover, it is caused by at least 147 mutations and many single-nucleotide polymorphisms on a single gene. Here we have detailed the causes and current clinical treatments of KD. We have highlighted current in vitro and in vivo models for the diseases. Finally, we reviewed current approaches to transport particles across the BBB for the treatment of neurological disorders (specifically in the context of neurodegenerative and lysosomal storage disorders), and provided an overview of gene therapy, enzyme replacement therapy, and small molecule delivery in the context of treating KD.

Taken comprehensively, these studies provide some key insights towards the complicated overall picture of nanomedicines for the treatment of neurological disorders. These include the need to consider particle physico-chemical properties in the context of their delivery route: Considering intravenous delivery, higher aspect ratio particles (e.g. rods or discs) are better able to marginate in the cerebral vasculature, but may need “help” crossing the BBB due to their shape/size. Likewise, small, highly PEGylated rod-shaped particles may be better able to penetrate mucus for nasal delivery. High density PEGylation of particles is also tied to improved particle penetration in the brain parenchyma. It is apparent that the use of auxiliary methods of delivery (e.g. MRI-FUS, CED) are critical for maximizing particle distribution to the brain, but must be balanced with potential complications due to the invasive nature (e.g. with CED). Finally, there are a number of targeting ligands that may facilitate nanoparticle transport over the BBB, but the clinical efficacy of such targeting approaches in treating KD remains largely untested. While clinical trials are in place testing various viral vectors for treating KD, as well as small molecules for remyelination, the potential of nanomedicine for improving the treatment of KD is needs to be explored. This includes pre-clinical (in vivo) studies to evaluate the potential of nanomedicines to cross the BBB and deliver gene therapies, enzymes supplements, or small molecules. Thus, while the biological target of KD is clear, the successful treatment of KD is not so simple. Any successful therapy will need to have persistent results/effects, and to date there is no cure for this devastating disease. Systematic studies evaluating nanomedicines to deliver genes, enzymes, or drugs to treat KD are needed to fully evaluate if nanocarriers can facilitate in its treatment. In a more expansive context, these lessons can be further applied to numerous disorders affecting the brain and CNS.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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