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# Experimental Therapies in the Murine Model of Globoid Cell Leukodystrophy

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## Abstract

Globoid cell leukodystrophy (GLD), also known as Krabbe disease, is a rapidly progressing childhood lysosomal storage disorder caused by a deficiency in galactocerebrosidase (GALC). GALC-deficiency leads to the accumulation of galactosylsphingosine (psychosine), a cytotoxic lipid especially damaging to oligodendrocytes and Schwann cells. The progressive loss of cells involved in myelination results in a dysmyelinating phenotype affecting both the central and peripheral nervous systems. Current treatment for GLD is limited to bone marrow or umbilical cord blood transplantation. However, these therapies are not curative, and simply slow the progression of the disease. The Twitcher (Twi) mouse is a naturally-occurring, biochemically faithful model of human GLD that has been used extensively to study GLD pathophysiology and experimental treatments. In this review, we present the major single and combinatorial experimental therapies targeting these and other aspects of murine GLD. The overwhelming evidence suggests that even with the best available molecular tools, targeting a single pathogenic mechanism provides only minimal clinical benefit. More recently, combination therapies have demonstrated the potential to further advance GLD treatment by providing synergistic increases in lifespan. However, such therapies must be designed and evaluated carefully, as not all combination therapies yield such positive results. A more complete understanding of the underlying pathophysiology and the interplay between various therapies holds the key to the discovery of more effective treatments for GLD.

### Keywords

Krabbe disease; globoid cell leukodystrophy; galactocerebrosidase; lysosomal storage disease; gene therapy; bone marrow transplantation



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## Globoid Cell Leukodystrophy

Globoid Cell Leukodystrophy (GLD), also known as Krabbe Disease, is a rare autosomal recessive lysosomal storage disorder that results from the deficiency of the enzyme galactocerebrosidase (GALC). GALC removes galactose from the terminal end of galactosylated sphingolipids, including galactosylsphingosine (psychosine), a cytotoxic lipid that is exclusively degraded by GALC. The accumulation of psychosine in GALC-deficient cells leads to the clinical sequelae observed in patients with GLD.

Because oligodendrocytes are particularly susceptible to psychosine buildup, patients with GLD exhibit demyelinating phenotypes. In infantile GLD, the most common form of GLD, symptom onset typically occurs at five months of age, followed by rapid disease progression. Patients experience intractable seizures, central and peripheral nervous system (CNS and PNS) involvement, sensory loss (blindness and deafness), profound neuroinflammation, and rapid psychomotor deterioration. Death typically occurs at 2–4 years of age.

Currently, the only treatment for GLD is bone marrow (BM) or umbilical cord blood transplantation, which have been shown to slow the progression of GLD, but not to cure the disease. While post-transplant patients have a more indolent course, progressive neurologic deterioration still occurs, and quality of life is diminished. Furthermore, transplantation is clinically effective only when given prior to symptom onset.[1,2] While newborn GLD screening is available in some states, most patients in states without newborn GLD screening are diagnosed after symptom onset, and thus would be poor candidates for transplant. Due to these limitations, there is a great need for more effective therapies.

## The Twitcher Mouse

The Twitcher (Twi) mouse is a spontaneously arising murine model of GLD that has a mutation at position 1017 of the *GALC* gene, which creates a stop codon and results in nonsense mediated decay of the mRNA.[3] Clinical progression, biochemical and histological features of the Twi mouse closely align with those of human GLD patients. Symptom onset occurs at postnatal day (PND) 21, followed by a rapid progression of disease. Affected mice experience demyelination of the central white matter tracts and peripheral nerves, weight loss, hind limb ataxia, kyphosis, and severe tremor. Death occurs at approximately PND 36.[4] Because it closely mimics the human disease, the murine model has been invaluable for the study of both the characteristics and the potential treatments of GLD.

Here we present a thorough, but not exhaustive review of the major single (Table 1) and combination (Table 2) therapies that have been attempted in the treatment of murine GLD. We also discuss their putative mechanisms, and their respective strengths and weaknesses.

## Single Modality Therapies for Murine GLD

### Cell-Mediated Therapies

Bone marrow transplantation (BMT) from a congenic, enzymatically normal donor mouse was thought to be effective for GLD because it provided a continuous source of cells

(microglia/macrophages) that could migrate into the brain and secrete the deficient enzyme. *In vitro* experiments have shown that secreted GALC can be taken up by enzyme-deficient oligodendrocytes, Schwann cells, and astrocytes via lysosomal enzyme-targeting receptors at the cell surface.[5–7] While this process, known as cross-correction, can facilitate the distribution of functional enzyme to mutant cells throughout the body, the extent to which this occurs differs greatly for different tissues. Significantly, GALC enzyme levels in the CNS only increased to 15% of normal donor levels following complete hematopoietic engraftment.[8] Incomplete correction of the CNS enzyme deficit may be the reason why Twi mice treated with BMT experience only a minor increase in lifespan to ~80 days. In addition, BMT provides no significant improvement in behavioral assays measuring limb strength[9–11], despite histological evidence of peripheral nerve remyelination.[8,12,13]

It has been hypothesized that hematopoietic stem cells (HSCs) overexpressing GALC may more effectively treat murine GLD. Attempts to overexpress GALC in murine HSCs were unsuccessful due to transgene toxicity specific to HSCs, but not their differentiated progenitors. More recently, GALC overexpression was induced in differentiated HSC progeny by using an HSC-specific miRNA to inhibit GALC expression in HSCs, but not their progeny.[14] Transplantation of the transduced HSCs into a murine model of GLD with <5% residual GALC activity increased lifespan to ~88 days, compared to ~50 days for untreated mutant mice and ~63 days for mutants receiving non-transduced GALC+/+ donor bone marrow. While the clinical benefits of donor hematopoietic GALC overexpression are mild, this study demonstrates the feasibility of autologous BMT using genetically modified autologous HSCs, which would eliminate the risk of graft rejection.

Mesenchymal stem cells (MSC) are derived from BM and adipose tissue, and have the potential to differentiate into adipose, muscle, and neuronal lineages.[15,16] Like cells of hematopoietic origin, MSCs also produce and secrete GALC. In addition, several studies have shown that they have immunosuppressive capacities[17–23], which may enable them to suppress the chronic neuroinflammation observed in the GLD brain. MSC transplantation does not require host BM ablation, and can be injected directly into the striatum, thereby more directly targeting the CNS. While injected MSCs decreased neuroinflammation in Twi mice, clinical measures of disease progression showed only minimal improvements.[24] Importantly, there was no increase in GALC activity in the brain, suggesting that the underlying enzymatic deficit remained uncorrected.[25] Additional studies using systemically delivered MSCs showed an increase in Schwann cell precursor proliferation and axonal density in the PNS[26,27], which correlated with only mild behavioral improvements.[28] Persistent psychosine buildup in the sciatic nerve in treated Twi mice[25] suggests that MSCs may not be effective as a single therapy because they don't alleviate the buildup of the toxic metabolite.

Neuronal stem cell (NSC)-directed gene therapy is a technique that involves injecting NSCs transduced with a recombinant, GALC-encoding viral vector directly into the brain. Theoretically, the overexpression and subsequent secretion of GALC allow for increased cross-correction of GALC-deficient cells, while the NSCs themselves can differentiate and replace damaged neuronal cells. Intrastriatal injection of recombinant retroviral-[29] and lentiviral-transduced[30] NSCs increased Twi lifespan to ~45 days and ~53 days, respectively.

Interestingly, control Twi mice treated with non-transduced WT NSCs had a similar increase in lifespan despite having much lower levels of GALC activity[29], This suggests either that GALC overexpression in the brain is not as critical as previously thought, or there are untargeted aspects of disease that limit the effectiveness of GALC overexpression.

## Enzyme Replacement Therapy

Enzyme replacement therapy (ERT) directly addresses the enzyme deficiency in GLD. Single dose intracerebroventricular (ICV) administration of GALC increased the Twi lifespan to an average of ~50 days.[31] This mild clinical effect may be due to enzyme turnover, as GALC enzyme is undetectable at the moribund stage.[32] Repeated enzyme injections are necessary to maintain a long-term functional pool of GALC enzyme. While repeated intracranial (IC) injections are infeasible, delivery of enzyme through a chronic port or by an intrathecal (IT) route is a possibility.

Repeated recombinant GALC delivery via intraperitoneal (IP) injections increased the Twi lifespan from ~40 to ~47 days, improved gait, and attenuated early-onset failure to thrive.[33] However, IP-injected GALC cannot cross the blood-brain barrier (BBB), and brain psychosine levels were 700-fold greater than WT levels in treated Twi mice. Because of the inability to penetrate the blood-brain barrier (BBB), and the infeasibility of repeated IC enzyme delivery, ERT is currently not available.

## Viral-Mediated Gene Therapy

Viral-mediated gene therapy utilizes a replication-defective virus to deliver the *GALC* cDNA to GALC-deficient cells in affected Twi mice, and has the potential to mediate long-term expression of the deficient enzyme. Of the viruses that have been tested in the treatment of murine GLD[5, 34–38], recombinant adeno-associated viruses (AAVs) have shown the most potential for the treatment of the neurological sequelae.

The AAV capsid protein determines the tropism and thus plays a large role in determining treatment efficacy. Previous studies have shown that the AAV5 capsid protein is more effective at transducing neurons than the first generation AAV2 capsid, inducing long-term GALC expression levels many-fold higher than WT levels in the Twi brain.[39] Correspondingly, AAV2/5-GALC increased median lifespan to ~63 days, and improved performance on neurobehavioral assays in Twi mice when delivered to the cerebellar vermis, cortex, and hippocampus.[40] Further improvements in lifespan and behavior were observed if an IT injection was added to the IC injections.[10]

More recently, AAVrh10 has been shown to have the highest long-term expression levels.[41] Twi mice treated with ICV, intracerebellar, and two intravenous (IV) injections of AAVrh10-GALC spaced one week apart had a mean life span of 120 days, with significant neurobehavioral improvements. Histologically, there is a significant reduction of neuroinflammation and improved CNS myelination in treated mice. However, foamy macrophage infiltration appears in treated animals starting at ~PND 60, and increases as the mice age, suggesting that disease progression still occurs, albeit at a slower rate.[42] Despite

this, AAVrh10-GALC gene therapy is, to date, the best single therapy in the treatment of murine GLD.

### Substrate Reduction Therapy

Substrate reduction therapy (SRT) utilizes small molecule compounds to inhibit the synthesis of galactosylated lipids, including psychosine. Genetic evidence that this type of therapeutic approach may be beneficial was first observed in Twi mice that only had one functional copy of the galactosylceramide synthase gene.[43] L-cycloserine is an irreversible inhibitor of 3-ketodyhydrosphingosine synthase, one of the enzymes required for psychosine synthesis, and low-dose administration to rats decreased levels of psychosine in brain myelin.[44] Repeated subcutaneous administration of L-cycloserine in Twi mice decreased astrogliosis and increased the mean lifespan to ~57 days.[45] Due to its inhibition of an enzyme that is upstream in the pathway of cerebroside synthesis, L-cycloserine also inhibits the synthesis of other cerebrosides integral for normal function. Thus, it has a relatively narrow safety window and its use will likely be limited to proof-of-concept experiments.

### Anti-Inflammatory Therapies

Neuroinflammation consisting of increases in immune cell activation and proinflammatory cytokines is a prominent component of GLD in both humans[46,47] and mice[48–53]. Elevated cytokines include TNF-alpha, a major regulator of immune cell activation. However, treatment of Twi mice with ibudilast, a phosphodiesterase inhibitor that decreases TNFα production in the brain, did not prolong lifespan but did delay weight loss.[52] Another group compared the levels of brain inflammatory markers after treatment with various anti-inflammatory medications. Indomethacin increased Twi lifespan to a median of ~65 days, and the observed increase correlated with a decrease in proinflammatory markers.[53] The mild improvement seen in these studies suggests that while neuroinflammation may play a prominent role in GLD, improvements will likely be mild if the primary enzyme defect is not addressed.

## Multi-Modality Therapies Targeting Murine GLD

To date, even the best single therapies for murine GLD only result in mild clinical improvements, suggesting that therapies targeting a single aspect of the disorder may not be very effective when given alone, but may have increased efficacy when given in combination.

### SRT and BMT

The first combination therapy attempted for murine GLD was BMT and SRT using L-cycloserine SRT. Combination-treated Twi mice experienced improved weight gain, decreased neuroinflammation, and a synergistic increase in lifespan to ~112 days[54], suggesting that combination therapies do indeed have a greater potential to treat murine GLD than single therapies.

### VEGF+BMT

One of the complicating factors in treating a predominantly neurologic disease is the relative impermeability of the BBB. The neonatal BBB can be temporarily rendered more permeable in response to IV administration of VEGF.[55] Combining IV VEGF administration and BMT without conditioning radiation led to a significant increase in Twi lifespan that was comparable to BMT with conditioning radiation. This suggests that increased infiltration of donor cells into the CNS in the absence of conditioning radiation enhances efficacy.

### Anti-oxidant + Anti-inflammatory Therapies

A diet-based therapy that eliminates galactose intake and increases antioxidant intake has been attempted in Twi mice.[56] This combination increased the mean lifespan to ~48 days, delayed tremor onset, and improved locomotor skills. *In vitro* studies confirmed that the antioxidants decreased oligodendrocyte apoptosis in the presence of psychosine. Unfortunately, confirmatory *in vivo* measurements of reactive oxygen species (ROS) were not performed. This becomes important since a more recent study showed that antioxidant therapy reduced markers of oxidative stress *in vivo* but had little effect on disease progression.[57] In the same study, combining the antioxidant N-acetyl cysteine (NAC) with BMT showed no significant improvements in lifespan or behavioral assays. Taken together, these studies suggest that ROS may be sufficiently downstream in the pathology for it to be an effective therapeutic target. Despite this, diet-based therapy is the least invasive treatment being studied, and additional research needs to be performed to determine whether dietary intervention will enhance the effects of more invasive procedures.

### ERT+BMT

One of the first studies directly targeting the two major components of murine GLD, enzyme deficiency and neuroinflammation, combined CNS-directed ERT with BMT. An additive increase in lifespan to 55 days was observed in treated Twi mice.[32] The absence of synergy in this particular treatment combination is most likely due to the relatively short *in vivo* half-life of GALC. The levels of GALC increased dramatically immediately post-injection, however, GALC activity was undetectable by PND 36.

### Viral-mediated Gene Therapy and BMT

Intravenous lentiviral-mediated gene therapy and BMT without conditioning radiation in newborn Twi mice led to a synergistic increase in lifespan to a median of ~75 days. Importantly, there was a delay between lentiviral administration and GALC expression, which coincided with a buildup of psychosine. Psychosine levels decreased when GALC expression was detected.[58] These results demonstrate the importance of *in vivo* characterization of viral distribution and enzyme expression during various stages of GLD progression.

Intracranial injection of recombinant AAV vector has also been studied in combination with BMT. A synergistic increase in average lifespan to ~104 days, body weight stabilization, and improved performance in the rotarod motor function test were observed. However, neurobehavioral performance declined as disease progressed.[11] The mechanisms leading to

the synergy observed in the AAV5/BMT study are only partially understood. It is known that CNS-directed AAV-mediated gene therapy supplies a persistent source of GALC activity to the brain.[59] However, the role that BMT plays is not fully understood. BMT appears to play an anti-inflammatory role in the treatment of murine GLD.[59–63] Flow cytometric analyses of cells of hematopoietic lineage in the brain showed an increase in CD45+ and CD11b+ cells in untreated Twi mice, indicating CNS infiltration by macrophages and microglia. Twi mice CNS-directed gene therapy alone had comparable increases in CD45+ and CD11b+ cells, as well as a significant influx of CD4+ and CD8+ T lymphocytes. Interestingly, addition of BMT to CNS-directed gene therapy decreased the levels of CD45+, CD11b+, CD4+, and CD8+ cells to WT levels, suggesting that BMT ameliorates both the disease- and AAV-specific neuroinflammatory responses. Immunohistochemistry also showed a decrease in astrocytosis and CD68+ activated macrophages/microglia.[10] These data are consistent with previous reports showing that Twi mice who received BMT experienced a decline in proinflammatory markers.[53, 63] Taken together, these data suggest that the immunomodulatory effect of BMT may be one mechanism contributing to the synergy observed when combined with CNS-directed gene therapy.

Compared to IC administration of recombinant AAV and BMT, addition of IT administration of recombinant AAV further increased the synergistic effect of the combination treatment to an unprecedented degree. Median lifespan of combination-treated Twi mice increased to ~123 days, with some mice living as long as 282 days.[10] These results confirm that therapies targeting multiple aspects of GLD can drastically improve disease outcome. Behavioral data support this conclusion: combination-treated Twi mice performed at near-WT levels in the accelerating rotarod until ~PND 140, nearly four months after untreated Twi mice would have died.[10] Interestingly, there were no improvements in the wirehang test for the combination-treated, BMT-only, or AAV2/5-only treated mice, regardless of method of recombinant AAV delivery. Because wirehang assays for limb strength and coordination, these results suggest that neither IC or IT gene therapy, nor BMT effectively targets PNS disease. Treatment modifications, including using a better viral capsid (i.e. AAVrh10 or AAV9) or adding other therapies that better target the PNS, may lead to improvements In the PNS disease.

## Conclusion

Ever since Twi mice were first described in 1980, numerous therapies have been attempted. However, none of the single treatments, even those that target the underlying genetic deficit, have been able to effectively treat the disease. Even the latest generation gene transfer vectors cannot prolong lifespan beyond 120 days, a small fraction of the 2–3 year WT lifespan. Characterization of the Twi mouse has identified multiple pathogenic mechanisms including, the underlying genetic deficit, psychosine accumulation, increased ROS, and neuroinflammation, to name just a few. The current data suggest that these different pathogenic mechanisms cannot be universally targeted by any single therapy alone. However, targeting different pathogenic mechanisms with rational combinations of therapies may have the greatest potential in addressing this complex neurodegenerative disease.

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## Table 1

Single Therapies for Murine GLD

| Therapy | Putative Mechanism | Lifespan of Treated GALC−/− Mutants | References |
|---|---|---|---|
| Bone Marrow Transplant | Cross-Correction<br>Immunomodulation | GALC+/+ Donor: 80 days* | 8, 9, 12, 13 |
| | | GALC-Overexpressing GALC−/− Donor: 88 days* | 14 |
| Mesenchymal Stem Cells | Immunomodulation<br>Replacement of damaged neuronal cells | 40 days | 23–27 |
| Enzyme Replacement Therapy | Short-term replacement of deficient GALC activity | Single-dose ICV administration: 50 days* | 31 |
| | | Weekly IP administration: 47 days* | 35 |
| Viral-Mediated Gene Therapy | Long-term replacement of deficient GALC activity | AAV2/5-GALC IC administration: 63 days | 40 |
| | | AAV2/5-GALC IC+IT administration: 71 days | 10 |
| | | AAVrh10-GALC adminsitration: 120 days* | 42 |
| NSC-Directed Gene Therapy | Cross-Correction<br>Replacement of damaged neuronal cells | Recombinant retroviral-GALC transduced NSCs: 45 days | 29 |
| | | Recombinant lentiviral-GALC transduced NSCs: 53 days* | 30 |
| L-Cycloserine | Substrate Reduction Therapy | 57 days* | 45 |
| Anti-Inflammatory Therapies | Immunomodulation | Ibudilast: Not reported** | 52 |
| | | Indomethacin: 65 days | 53 |

*Mean lifespans. All other life spans are represented by the median.

**Exact lifespan of treated Twi mice not reported, but was not significantly different compared to untreated mutants

## Table 2

Combination Therapies for Murine GLD

| Therapies | Lifespan | | | References |
|---|---|---|---|---|
| | Single | Combined | Response | |
| L-cycloserine | 56 days | 112 days* | Syngergistic | 54 |
| BMT | 51 days | | | |
| VEGF | ** | 50 days | Insignificant | 55 |
| BMT | 41 days | | | |
| Antioxidants | ** | 48 days* | N/A | 56 |
| Dietary Galactose Elimination | ** | | | |
| N-acetyl cysteine (antioxidant) | 37 days | 38.5 days | Insignificant | 57 |
| BMT | ** | | | |
| Single ICV ERT | 51 days | 59 days | Additive | 32 |
| BMT | ** | | | |
| IV Lentivirus-GALC | 50 days | 75 days | Synergistic | 58 |
| HSC administration without preconditioning | 60 days | | | |
| IC AAV2/5-GALC | 49 days | 104 days* | Synergistic | 11 |
| BMT | 44 days | | | |
| IC+IT AAV2/5-GALC | 71 days | 123 days | Synergistic | 10 |
| BMT | ** | | | |

* Mean lifespans. All other life spans are represent ed by the median

** Lifespan for the indicated control single-therapy condition not reported in the paper cited, but may be available in other papers (See Table 1)