# KRABBE DISEASE OR GLOBOID-CELL LEUKODYSTROPHY

**FOR PARTIAL FULFILLMENT OF B.Sc. BIOTECHNOLOGY**

**REVIEW**

**BY**


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# *CERTIFICATE*

**23/03/16**

This is to certify that **Mr. Diptanu Debnath**of **Third year, B.Sc. Biotechnology** has satisfactorily completed the REVIEW REPORT for fulfillment of Bachelor Degree is Biotechnology as prescribed in the syllabus of **Rajiv Gandhi Institute of IT and Biotechnology, B.V.D.U, Pune, in the academic year 2012-2013.**

Dr Shamim Shaikh Dr. G.D. Sharma

CandidateReview Guide Principal

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## 2. Abstract:

Krabbe disease, or globoid-cell leukodystrophy (GLD), is a progressive neurodegenerative disease which is caused by the absence or malfunctioning of the enzyme galactocerebrosidase (GALC) due to mutations caused in the gene which encodes for it. The prognosis of this disease is poor and affected individuals usually perish within the age of 5-6 years. This review is an attempt to summarise and have a general overview of the disease, its pathophysiology, the factors which cause it and the various attempts being carried out for the treatment of this disease.

In this review, an attempt has been made to cover the basic aspects of this disease, such as progression, pathophysiology, symptoms, etc. Also, the enzyme GALC has been scrutinised for its functioning and the various mutations in its gene which leads to its malfunctioning. Finally, an attempt has been made to bring into light the various attempts which are being made for developing a successful treatment of the disease as no known cure is present for this disease at this stage.

**Keywords:**GLD, Krabbe disease, lysosomal storage disease, Galactocerebrosidase, GALC, psychosine

## 3. Krabbe Disease: An Introduction

Krabbe disease (K. Krabbe, 1916)is a lysosome storage disease caused by the deficiency of the enzyme galactocerebrosidase (GALC). It was first described in 1916 by the Danish neurologist, Knud Krabbe. Krabbe who reported an uncommon form of familial diffuse brain sclerosis. GALC is responsible for the hydrolysis of galactolipids (lipid components of the myelin membrane), the major ones being galactocerebrosidase and psychosine.

The disease is assumed to be caused primarily due to the accumulation of the galactoshingolipid, psychosine. Surprisingly, psychosine is not the primary substrate of the effected enzyme, GALC.Psychosine accumulation leads to demyelination of the oligodendroocytes, which leads to severe neurological implications.The pathology of GLD is characterized by the destruction of oligodendrocytes, reduced myelin formation and the accumulation of globoid cells.

The prognosis of the disease is poor, and the affected individuals usually start showing the symptoms around the age of 6 months – 2 years. They soon succumb to the disease due to an increase in many directly-unrelated infections

Till date, no known cure is present for GLD. Most of the present treatment is based on stem cell therapy. But a lot of new methods are being theorised and attempted for the treatment of this disease.

## 4. Globoid Cell Luekodystrophy (GLD): The Disease

Krabbe disease is a lethal, progressive, neuro-degenerative disorder with no cure. It is characterized by the progressive demyelination and presence of globoid cells and hence is also called 'globoid-cell leukodystrophy'. While the disease is related to the mutations caused in the gene of the enzyme involved in it, it is inherited in an autosomal recessive manner (J.S. Won *et al.*, 2013).

Krabbe disease is caused by a genetic deficiency of the lysosomal hydrolase, galactosylceramide β -galactosidase (GALC;E.C. 3.2.1.46). GALC degrades galactosylceramide, a major component of myelin, and other terminal β-galactose–containing sphingolipids, including psychosine (D-galactosylsphingosine) (J.S. Won *et al.*, 2013).

Unlike other sphingolipid storage diseases, the primary substrate of the enzyme, galactosylceramide, does not get accumulated in the central nervous system as it can be degraded by GM1 ganglioside β -galactosidase (EC 3.2.1.23). However, psychosine is not hydrolyzed by GM1 ganglioside β –galactosidase.Hence, psychosine accumulates excessively in the brain of GLD patients. The increased psychosine levels in the neural tissue are believed to lead to widespread degeneration of oligodendrocytes (OLs) and to subsequent demyelination (J.S. Won *et al.*, 2013).

Psychosine is the primary component responsible for the destruction of oligodendrocytes and Schwann cells, which produce myelin. This results in demyelination of the central and peripheral nervous systems. Additionally, it causes activation of astrocytes and the formation of multinucleate globoid cells (macrophages with accumulations of galactocerebrosides), which are characteristics of the disease in the pathological examination (J.S. Won *et al.*, 2013).

### 4.1. The Symptoms of GLD:

There are two clinical forms of GLD: early onset infantile and late onset. The first form is more common (90% of cases) and symptoms generally begin before 6 months of life (71%); in the late onset form, 6% develop symptoms between 13 to 24 months, 3% between 25 and 36 months, and 1% after 5 years(T.S. Sano, 2012).

In the infantile form, the main symptoms are excessive crying, irritability, stiffness, seizures, and difficulty holding up the head. In the late onset form there are alterations in gait, motor development delay, stiffness, lossof vision, dysphagia, and seizures(T.S. Sano, 2012).

### 4.2. Epidemiology:

1. GLD is rare, with an incidence of 1/100,000 live births. It presents as a hereditary recessive autosomal disorder, with a deficiency of GALC due to the mutation of the GALC gene located in chromosome 14q31(http://www.ncbi.nlm.nih.gov/gene/2581)– there are also more than 60 known mutations in this gene.

   Most patients develop symptoms during the first months of life and the mean time between onset of symptoms and diagnosis is 5.3 months.The diagnosis is made by determination of GALC activity in isolated leukocytes or in skin fibroblast cultures(T.S. Sano, 2012). Values of GALC activity lower than 15 nmol/17h/mg protein confirm the diagnosis(T.S. Sano, 2012).

Other alterations found are proteinorachia; electroneuromyography with reduced rate of neural conduction. The EEG may demonstrate unspecific findings, consistent with diffuse cerebral distress; on the MRI, as the disease progresses, the white matter becomes hypoattenuated and atrophied, and there is a T2 hyperintense signal in the thalamus, corona radiata,and body of the caudate nucleus(T.S. Sano, 2012).

## 4.3. Prognosis:

Prognosis of the disease is poor. Patients evolve with progressive neurological deterioration until coma and death within and average of 24.1 months(T.S. Sano, 2012). Some factors worsen the prognosis, such as, onset of symptoms before 6 months of age and presence of threesymptoms:

- Stiffness
- Loss of vision, and
- Dysphagia.

The level of enzyme activity and the type of mutation are not related to prognosis.

Patients have usually been found to succumb to infections, which ultimately lead to death. The reason behind this increased rate of infections in still not clear. But it may be related to the impact GLD has on the immune system (A.A. Maghazachi, 2013).

## 4.4. Differntiation of Oligodendrocytes:

For the proper understanding of this disease, it is essential that one understands the process of formation, maturation and myelination of oligedendrocytes, to which the disease is directly related.

Myelin is a fatty white substance that surrounds the axon of some nerve cells, forming an electrically insulating layer. It is essential for the proper functioning of the nervous system. It is an outgrowth of a type of glial cell. The production of the myelin sheath is called myelination or myelinogenesis (J.S. Won *et al.*, 2013).

Oligodendrocytes are the myelinating cells of the central nervous system (CNS). They are the end product of a cell lineage which has to undergo a complex and precisely timed program of proliferation, migration, differentiation, and myelination to finally produce the insulating sheath of axons (J.S. Won *et al.*, 2013).

Myelination involves specific stagewise differentiation of proliferating OL precursor cells (OPCs) into post-mitotic OLs. Each differentiation stage is identified by morphological features and specific patterns of marker expression (J.S. Won *et al.*, 2013).

Differentiation of OPCs to myelin forming mature-OLs is prerequisite for myelination and is accompanied through four sequential stages (OPC, pro-OL, immature-OL and mature-OL stages). Among these stages, the onset of terminal differentiation at stage 3 is of particular interest because the immature-OLs at this stage start to express CGT for synthesis of galactosylceramide and other myelin lipid components (J.S. Won *et al.*, 2013).

Oligodendrocyte precursor cells (OPCs) are small round bipolar cells with proliferation and migratory potential and express OL specific transcriptional factors

(Olig 1 and Olig 2), cell surface ganglioside epitope A2B5, and platelet-derived growth factor receptor-α (PDGF-Rα) (J.S. Won *et al.*, 2013).

Pro-OLs extend multipolar short processes and start to express, in addition to early OPC markers, Sox17 and sulphatides recognized by O4 antibody. Immature-OLs are post-mitotic cells characterized by long processes, expression of CNPase (2′, 3′-cyclic nucleotide 3′-phospho-diesterase) and synthesis of galactosylceramide (J.S. Won *et al.*, 2013).

Mature-OLs extend myelin membranes and express myelin proteins such as proteolipid protein (PLP) and myelin basic protein(MBP). Among these stages, stage 3 is of particular interest because the immature-OLs at this stage synthesize substantial amount of myelin lipids including galactosylceramide and plasmalogens (J.S. Won *et al.*, 2013).

The galactosylceramide can be converted to psychosine by deacylation under deficiency of GALC activity. The CGT expression and thus enhanced CGT activity during this stage may also participate in increased synthesis of psychosine, in turn suggesting that psychosine may begin to accumulate at stage 3 of OLs differentiation and may affect terminal differentiation of immature-OLs. Therefore, the protection of differentiating OLs at stage 3 may be critical for prevention of impaired myelination and remyelination in Krabbe disease (J.S. Won *et al.*, 2013).

## 4.5. Neuromuscular Dysfunction in GLD:

Krabbe disease has an extremely high impact on the neuromuscular junctions. And while the entire method of this process is still not completely clear, it has to be understood that this is one of the major issues arising out of this disease.Studies carried out using the Twitcher mouse model of have revealed that there are key elements of a complex pathogenic mechanism affecting the function, structure, and growth of skeletal muscles in Krabbe disease (L. Cantuti-Castelvetri*et al*., 2015). There are signs of both presynaptic and postsynaptic defects in the neuromuscular junction. Psychosine, it has been observed, contributes to the pathology that leads to these muscular defects in GLD. Psychosine, it seems, impacts muscle pathology by decreasing the functioning and concentration of Protein Kinase B (L. Cantuti-Castelvetri*et al*., 2015).

Motor deficiency is a hallmark in all untreated Krabbe patients,whoundergohypokinesia,musclewasting,andatrophy. Perhaps the reason behind muscle atrophy in GLD is a consequence of dysfunctional NMJs rather than the complete physical loss of NMJ contact. For the time being, further studies need to be carried out down this line so as to better understand the process of neuromuscular dysfunction in GLD. Only then can a proper method for treatment, or at least reduction, of this state be put forward for those suffering from GLD (L. Cantuti-Castelvetri*et al*., 2015).

## 4.6. Impact of GLD on NKCs:

NKCs, or natural killer cells are an important part of the immune system. They, along with macrophages, neutrophils, etc. play a very crucial role in protecting the body against pathogens which invade the body. GLD has been found to impact the NKCs in the Twitcher mouse model of the disease, perhaps even inducing the apoptosis of the NKCs. In rats, psychosine has been found to induce the apoptosis of

the G6 glial cells *in vitro*. Psychosine also induces the apoptosis of MO3.13 cell line*in vivo*, which has been regularly used as a cell-based model for the study of GLD (A.A. Maghazachi, 2013).

As far as the mechanism of the induction of apoptosis due to psychosine is concerned, it has been found to bind to a heptahelical receptor named T-cell-death-associated-gene 8 (TDAG8), which is known to bind to heterotrimeric guanine nucleotide-binding proteins, G proteins. The glycosylated form of psychosine has also been found to bind to TDAG8 (A.A. Maghazachi, 2013). Human TDAG8 exists in normal tissues and is restricted to lymphoid organs, such as spleen and lymph nodes, as well as its expression in peripheral blood lymphocytes. Due to the presence of this receptor on NKCs, and psychosine's ability to bind to it, psychosine has been found to induce apoptosis and multinucleation among NKCs.

Psychosine induces the *in vitro* chemotaxis of NKCs as well as their accumulation in splenic white pulps in Twitcher mice. It also supports the induction of apoptosis, as has already been mentioned (A.A. Maghazachi, 2013). Along with NKCs, the presence of macrophages and dendritic cells, which play important roles as Antigen Presenting Cells (APCs), in spleen is also affected.

## 5. The Enzyme: Galactocerebrosidase:

Galactosylceramide β –galactosidase, also commonly known as β-galactosylceramidase or galactocerebrosidase(GALC;E.C. 3.2.1.46), is a lysosomal hydrolase which is present in oligodendrocytes (OLs). It is encoded for by the GALC gene, located in chromosome 14q31. GALC is a typical example of carbohydrate-active enzymes (CAZymes). It's classified under CAZy classification as glycoside hydrolase family 59 (GH59) (J.S. Won *et al.*, 2013).

GALC is synthesised and glycosylated in the Endoplasmic Reticulum (ER) – Golgi complex (ERGC). Then, it gets transported to the lysosome by the mannose-6-phosphate pathway. GALC is essential for normal catabolism of galactosphingolipids, including the principal lipid component of myelin, galactocerebroside. Sphingolipid degradation requires the combined action of water-soluble hydrolases and nonenzymatic sphingolipid activator proteins known as saposins.

The GALC gene encodes a lysosomal protein which hydrolyzes the galactose ester bonds of galactosylceramide, galactosylsphingosine, lactosylceramide, and monogalactosyldiglyceride (http://www.ncbi.nlm.nih.gov/gene/2581). Its main substrate is galactosylceramide. However, in the case of GLD, it's not the main substrate, but a secondary substrate, namely psychosine, which gets accumulated due to the missing or mutated enzyme. This is so because the enzyme $G_{M1}$-gangliosidase also is capable of degrading galactosylceramide, but isn't capable of degrading psychosine (J.S. Won *et al.*, 2013).

The optimum pH of GALC activity is between pH 4.5 and 5.0 consistent with its lysosomal localization.

### 5.1. GALC Structure (*Mus musculus*):

The human GALC structure consists of 669 amino acid residues. The structure of mouse GALC, which is an ortholog the human GALC, has an83% match with its human counterpart. The mouse ortholog has been extensively studied to understand the working of human GALC, mutations which may lead to GLD and its effect in GLD (J.E. Deane *et al.*, 2011).

The structure of mouse GALC, when refined to 2.1 Å, is shown to contain one molecule per asymmetric unit comprising residues 25 to 688. The overall fold comprises three domains:

- A central triosephosphate isomerase (TIM) barrel,
- A β-sandwich domain, and
- A lectin domain, which is a unique feature of GALC.

The first 24 residues of GALC encode the signal peptide for targeting to the ER. The residues 25–40 contribute two β-strands to the β-sandwich domain.The $(\beta/\alpha)_8$ TIM barrel is composed by residues41–337and lies at the centre of the structure. Residues 338–452 then form the remainder of the β-sandwich domain. The lectin domain is formed by residues 472–668. Residues 453–471 lie across one face of the structure stretching from the β-sandwich to the lectin domain. The interfacesformedbetweeneachofthedomainsofGALCarevery large: 22% (1;770 Å2) and 15% (1;480 Å2) of the solvent accessible surface areas of the β-sandwich domain and the lectin domain, respectively, are buried in their interfaces with the TIM barrel.Thus,thedomainsofGALCare notlikelytomove relative to each other (J.E. Deane *et al.*, 2011).

The central TIM barrel is composed of eight parallelβ-strands surrounded byα-helices. The connecting loops on theC-terminalside of the barrel (proximal to the lectin domain) are generally longer than those on the opposite side of the barrel (J.E. Deane *et al.*, 2011).

The β-sandwich domain comprises two twisted β-sheets with a similar topologytothatseeninotherglycosylhydrolasesexceptforaverylong loopthatwrapsoverthetopoftheTIMbarrel.Acysteineresidue in this loop, C378, forms a disulfide bridge with C271 in the TIM barrel (J.E. Deane *et al.*, 2011).

Acalcium ionisbound inthelectin domain ofGALCina pentagonal bipyramidal configuration. It lectin domain also contains a Calcium-binding site and a fold. The lectin domain of GALC also possesses structural similarity to galectin proteins and other carbohydrate-binding lectins.Residues N284, N363, N387, and N542 displayed electron density for glycosylation moieties, indicating the probable sites of glycosylation of the enzyme before it gets transported out to the lysosomes from the ERGC (J.E. Deane *et al.*, 2011).

## 5.2. Active Site:

The interactions and atomic distances between atoms of galactose and GALC identify E258 as the active site nucleophile and E182 as the proton donor. The average distance between the carboxyl oxygens of these catalytic residues is 5.0 Å, consistent with the retaining mechanism of enzymatic glycosidic bond hydrolysis in which the product retains the same stereochemistry as the substrate. In addition to the catalytic glutamates E182 and E258, several residues contribute to the formation of the substrate-binding pocket and form hydrogen bonds with the bound galactose molecule (J.E. Deane *et al.*, 2011).

## 5.3. Cleavage Site:

It has been found that GALC uptake into the lysosomes, after it has been released from the ERGC, is dependent on cleavage of GALC. During this process,

GALC is cleaved into two fragments, 50 kD and 30 kD. This is followed by its uptake into the lysosome. However, it has not been associated with GLD complications as the cleavage site lies quite far away from the active site of the enzyme.The cleavagesitelieswithin a loop of theβ-sandwich domain, far fromtheactive site.Following cleavage, it isextremely unlikely that the twofragments would dissociate given the very large buried surface area between them.Thus, in mutant forms of GALC, where the enzyme is not cleaved, it is incorrect targeting, with the side effect of failure to encounter processing enzymes,ratherthanlackofcleavagepersethatislikelytobethe critical deficit (J.E. Deane *et al.*, 2011).

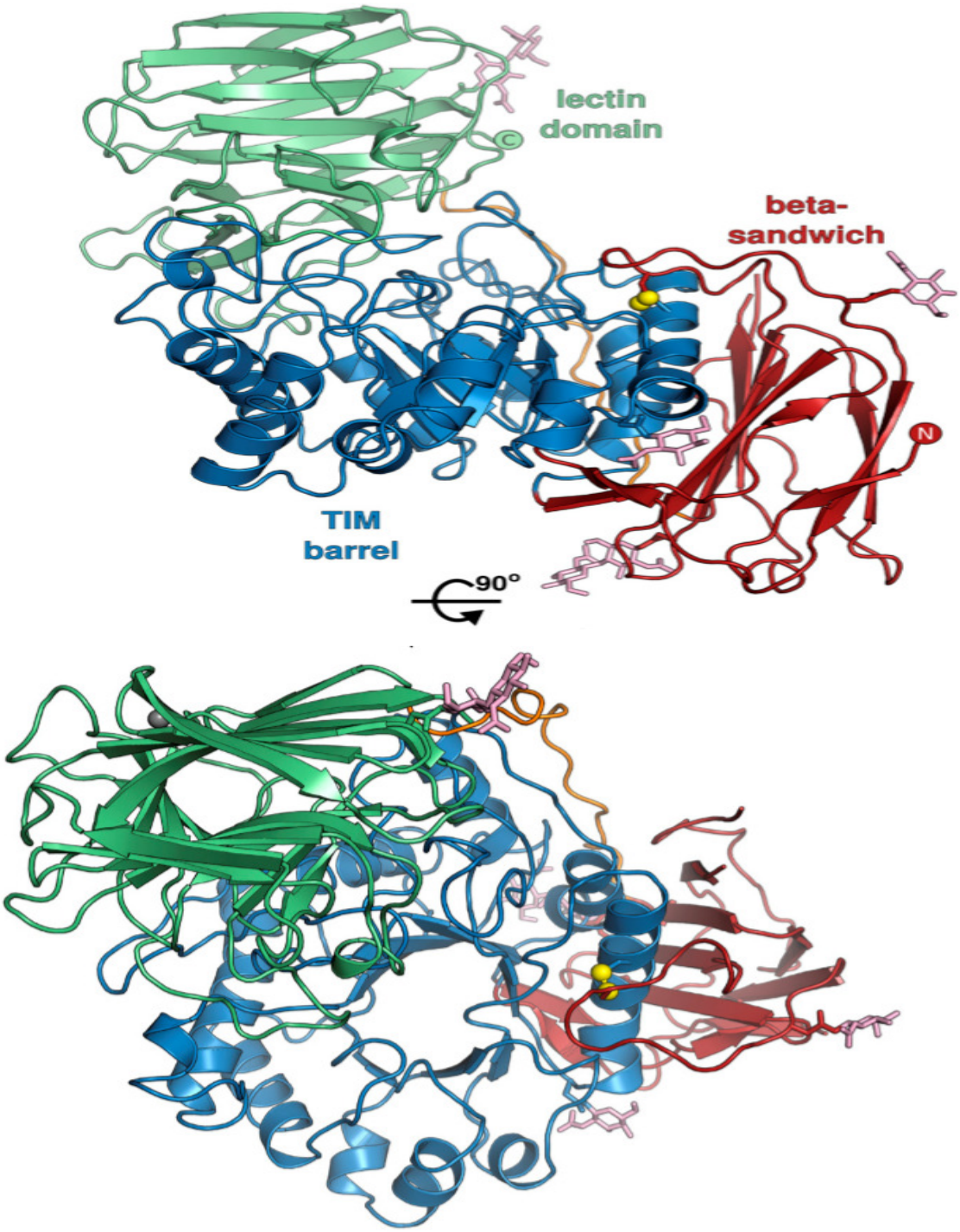


**Fig. 1.: Structure of GALC shown in two orthogonal views.** Ribbon diagram of GALC coloured by domain: β-sandwich (red), TIM barrel (blue), linker (orange), and Lectin domain (green). The disulfide bond (yellow spheres), calcium ion (gray sphere), and glycosylation moieties (violet sticks) are shown. The N and C termini are marked by labeled circles (Top)(J.E. Deane *et al.*, 2011).

## 5.4. Substrate Specificity:

GALC catalyses the hydrolysis of the galactosyl moiety from glycosphingolipids such as galactocerebroside and psychosine. The active site of GALC only accommodates galactose and is very specific. The overall fold of GALC is unchanged upon galactose binding, the core of the binding pocket being formed by the long loops on the C-terminal face of the TIM barrel as has been observed for other enzymes containing TIM barrels. However, loops from both the β-sandwich and lectin domains also contribute to the substrate-binding pocket. The position and orientationofgalactosein the activesiteisunambiguous.

Many lipids also contain glucose. However, GALC specifically recognizes galactose-containing lipids. Galactose and glucose differ only in the position of a hydroxyl group at one carbon (C4, Fig. 2F). In the galactose-bound structure of GALC, this hydroxyl forms a hydrogen bond with T93: a residue with unusual backbone dihedral angles (φ¼ = 139°, ψ ¼ = −48°) that aremaintainedintheabsenceofgalactose,indicatingthisresidue confersspecificityratherthanundergoinganinducedfit (J.E. Deane *et al.*, 2011).

Glucose is not compatible with the substrate-binding pocket as the position of its hydroxyl group would clash with residue W291. The conformation of W291 is stabilized by a hydrogen bond with the backbone carbonyl of G47 (J.E. Deane *et al.*, 2011).

The binding of galactose is further stabilized by hydrogen bonding to R380 at the tip of the long loop that stretches from the β-sandwich domain. This loop is secured in position by the disulfide bond formed between C271 in the TIM barrel and C378 in the loop. The position of the side chain of R380 alters slightly upon galactose binding to accommodate the ligand (J.E. Deane *et al.*, 2011).

## 5.5. Conformation of active site during substrate binding:

R380 is a residue lying in the active site of GALC. This residue plays a major role in the catalysis of the substrate. Mutation of this residue to tryptophan or leucine has been found to cause Krabbe disease. During catalysis, the side chain of the catalytic acid/base residue E182 can adopt two distinct conformations. In addition, we observe that the noncatalytic substrate-binding residue R380 also alternates between two different conformations throughout the catalytic cycle. Upon binding of substrate, the side chain of R380 moves to accommodate the substrate and forms a hydrogen bond with the galactosyl 6-hydroxyl. Upon formation of the covalent intermediate, R380 moves back to a conformation similar to that seen in the unliganded enzyme. However, in this complex, two ordered water molecules that were not present in the unliganded structureappear intheactive site hydrogen-bondedtobothR380and E182. To complete the catalytic cycle, E182 activates one of these waters to attack the enzyme-bound intermediate. In the enzyme–product complex, the side chain of R380 is again hydrogen bonded to the galactose 6-hydroxyl, as seen in the enzyme–substrate complex (J.E. Deane *et al.*, 2011).

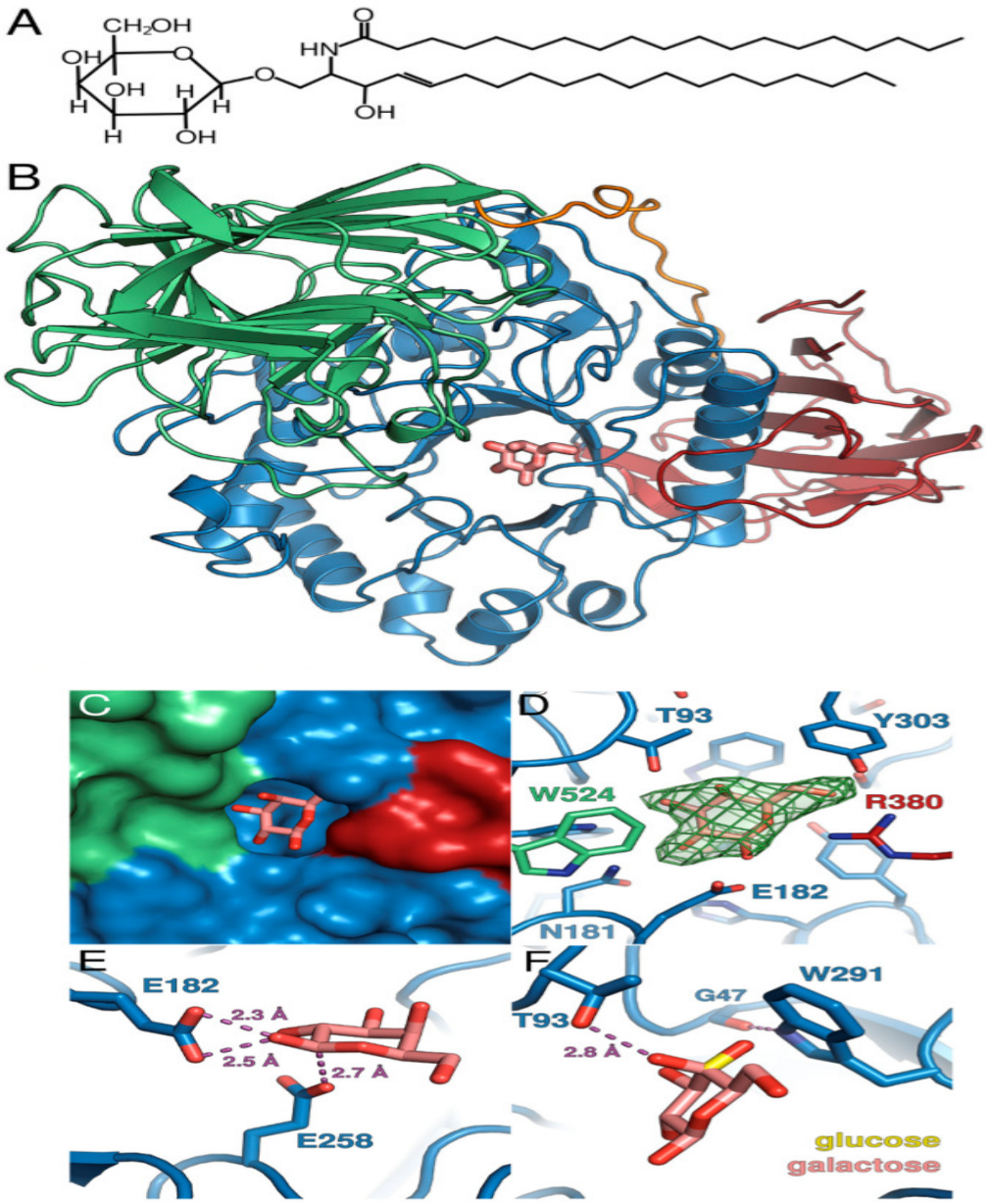
A
CH2OH
OH
O
HN
O
OH
H
H
H
H
OH
OH
B
C
D
T93
Y303
W524
R380
E182
N181
E
E182
2.3 Å
2.5 Å
2.7 Å
E258
F
W291
G47
T93
2.8 Å
glucose
galactose

**Fig. 2. : Substrate-binding site of GALC.** (A) Schematic diagram of the GALC substrate galactocerebroside. (B) Ribbon diagram of GALC illustrating bound galactose (pink sticks) with domains colored as in Fig. 1. (C) Surface representation of GALC zoomed in on the galactose-binding site, colored and oriented as in B.(D) Unbiased difference (FO-FC) electron density (green mesh) corresponding to galactose bound to GALC. (E) Active site of GALC illustrating the relevant atomic distances (purple dashed lines) between the bound galactose (pink sticks) and the proposed catalytic residues: the nucleophile (E258, blue sticks) and the proton donor (E182, blue sticks). (F) Substrate specificity for galactose- (pink sticks) rather than glucose (yellow sticks) containing glycolipids is conferred by W291 and T93 (blue sticks)(J.E. Deane *et al.*, 2011).

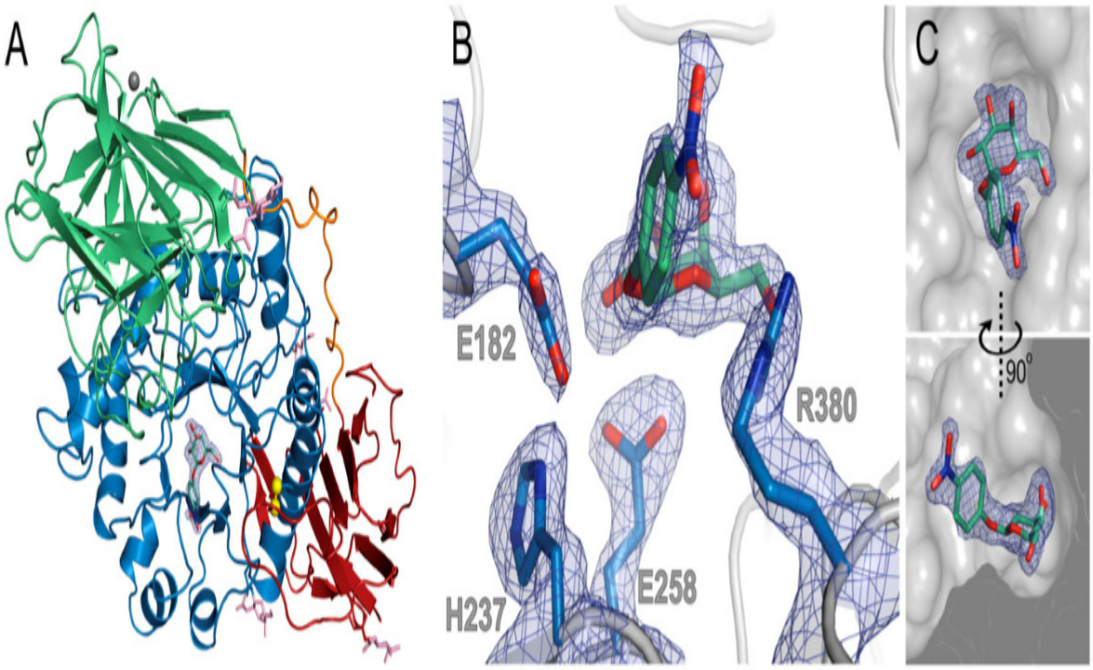


**Fig. 3. : Structure of the wild-type GALC enzyme in complex with substrate.** (A) Ribbon diagram showing the overall structure of GALC with the substrate 4NβDG (a synthetic substrate) bound in the active site. The electron density (2FO-FC contoured at 0.25 e–/Å3, blue) is shown for uncleaved substrate bound in the active site pocket of GALC. Surface glycans (pink sticks) are shown. (B) Detail of the GALC active site with bound, uncleaved substrate showing active site residues (sticks) and electron density (as above). (C) Surface representation of the GALC active site (gray) with the substrate and electron density shown (as above) (C.H. Hill *et al.*, 2013).

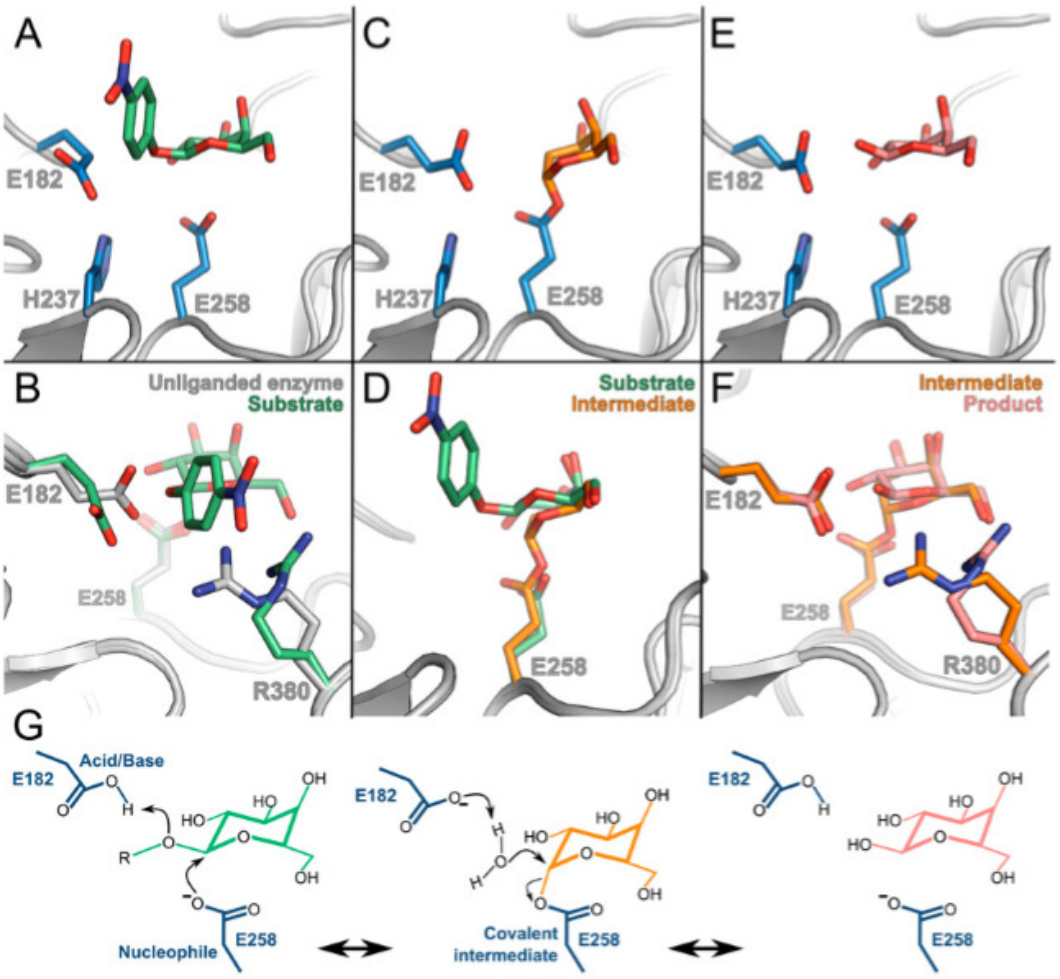


**Fig. 4. : Structures of the GALC active site illustrating conformational changes along the reaction coordinate.** (A) Wild-type enzyme in complex with substrate 4NβDG (green). (B) Overlay of the GALC active site residues in the absence (gray) and presence (green) of substrate. (C) Covalent intermediate structure illustrating D-galactal (orange) covalently attached to the catalytic nucleophile. (D) Movement of E258 and the pyranose ring between substrate binding (green) and covalent linkage with the inhibitor D-galactal (orange). (E) Enzyme–product complex formed following extended incubation with substrate illustrating catalytic activity of GALC in crystallo.( F) The two different conformations of the R380 side chain in the covalent intermediate (orange) and product (pink) complexes. (G) Schematic representation of the proposed retaining two-step glycosidic bond hydrolysis reaction(C.H. Hill *et al.*, 2013).

## 5.6. Mode of Action:

GALC-mediated degradation of galactolipids requires the activity of saposins, sapA and possibly also sapC. Two mechanisms of action for saposins have been postulated. Thefirstsuggestssaposinsextractthelipidsfrombilayerstoform water-soluble lipid–protein complexes that present the substrate to the appropriate enzyme. The second involves the binding of enzyme at the bilayer surface where saposin molecules facilitatetheaccesstosubstratebydistortingthebilayer.It may be possible that, during GALC activation, both sapA and sap C will be involved via these different mechanisms (J.E. Deane *et al.*, 2011).

## 5.7. Effect of mutations on GALC:

While many mutations have been identified causing various effects on the functioning and structure of GALC, there are about 60-70 which are related to GLD. Among them, a large proportion of missense mutations may lead to mistargeting or early degradation of the protein.Mistargeting could occur at several stages in the GALC processing pathway, including failure to transit from the ER to Golgi due to misfolding, and blockage of trafficking to the lysosome due to altered binding to the M6P receptor (J.E. Deane*et al.*, 2011). In addition, if mutations affect the binding of GALC to essential activating factors in the lysosome (including saposins) then, despite being correctly processed and localized, GALC may not be able to access substrate for efficient glycosphingolipid cleavage.

Among the various missense mutations related to Krabbe Disease, nearly 70% lead to the modification of residues buried deep within the structure of the enzyme which lead to instability or misfolding of the enzyme, ultimately leading to premature degradation. These mutations are spread around and effectalmost all the domains of the enzyme.

Mutations that are likely to result in severe misfolding include E114K and S257F in the TIM barrel, L364R and W410G in the β-sandwich domain, and G537R and L629R in the Lectin domain. The mutation, L629R has indeed been found to be related to accumulation of GALC in the ER and absence of secretion of the enzyme by cells or detection of it in lysosomes (J.E. Deane *et al.*, 2011).

Within the ERGC, GALC undergoes modification by the addition of glycans to specific asparagine residues. This N-linked glycosylation is essential for the correct transportation of GALC including binding to the M6P receptor. Among patients of Krabbe disease having Arab ancestry, the mutation D528N has been found to introduce a new glycosylation site in GALC. This leads to a mutated hyperglycosylated form of the enzyme which is then not taken up by the cells and hence not present in lysosomes.

One more effect that some of the mutations may have is to interfere with binding to activating factors, such as saposins, instead of affecting the residual enzyme activity, processing, or localization of GALC. The Krabbe disease-related mutation E215K is perhaps an example.This mutation confers an opposite charge on the same face as the substrate-binding pocket suggesting that the mechanism of

disease for this mutation will involve theperturbationofabindingfaceforanactivatingfactor that is essential for efficient in vivo glycolipid metabolism (J.E. Deane *et al.*, 2011).

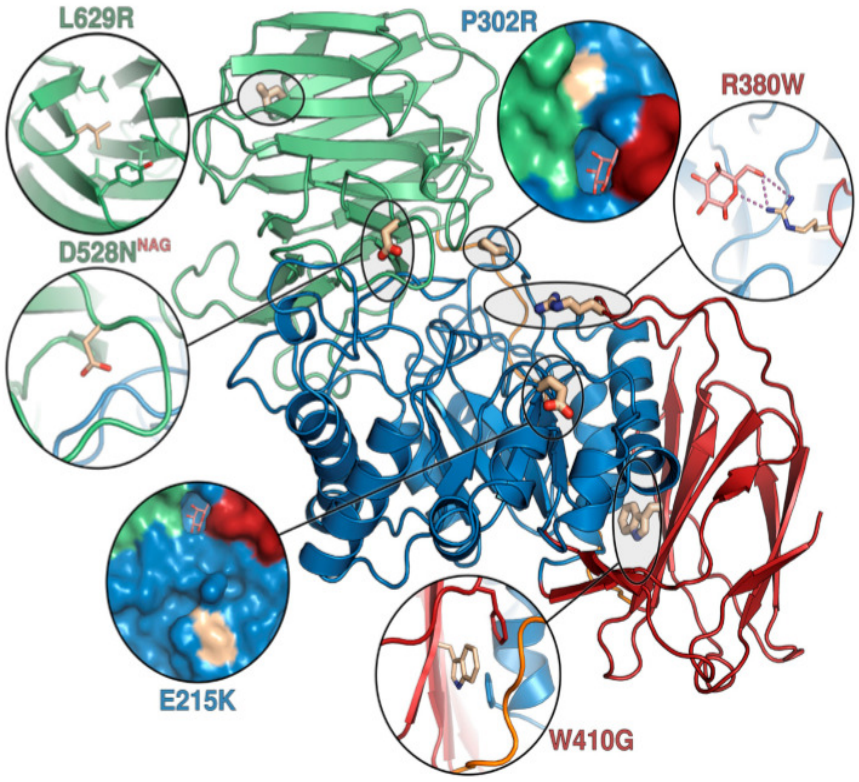


**Fig. 5.: Disease-associated mutations of GALC.** A selection of residues that are mutated in Krabbe disease are shown as sticks (beige) on the structure of GALC. For each residue a relevant view is provided (Insets) illustrating the surrounding region of the structure that would be perturbed by the mutation. Because of the insertion of a residue in the human sequence at position 507, residues 528 and 629 correspond to 527 and 628, respectively, in the mouse structure(J.E. Deane *et al.*, 2011).

## 6. Psychosine

D-galactosylsphingosine, also known commonly as Psychosine, is a β-galactose – containing spingholipid produced in the oligodendrocytes (OLs) of the brain. Pscychosine accumulation in OLs has been considered to be the primary reason behind Krabbe disease. It is the secondary substrate of the enzyme galactocerebrosidase (GALC), impairment of which leads to GLD.

Psychosine is synthesized by the action of UDP-galactose ceramide galactosyl transferase 8 (CGT or UGT8; E.C. 2.4.1.45). In the brain, CGT is primarily expressed in the OLs, in which it catalyzes the transfer of galactose to ceramide and sphingosine to form galactosylceramide and psychosine, respectively. Psychosine is a lysolipid having detergent like properties. Therefore, its excessive accumulation over threshold in GALC deficient cells can trigger membrane destabilization leading to cell lysis. Concentrations as high as 20 μM have been found to be lethal to OLs (J.S. Won, 2013).

Under normal conditions, both galactosylceramide and psychosine in the brain is maintained at very low levels by the activities of galactosylceramide β -galactosidase (GALC;E.C. 3.2.1.46), also known as galactocerebrosidase. However, psychosine continues to accumulate under GALC-deficient conditions and may account for as much as 50% of cerebrosides. Whereas,galactosylceramide gets degraded by $G_{M1}$ ganglioside β -galactosidase (EC 3.2.1.23).

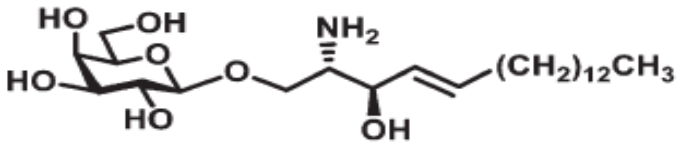


**Fig. 6. : Psychosine**(J.A. Hawkins, 2013)

## 6.1. Role of Psychosine in GLD

In a group of experiments carried out at the Medical University of South Carolina, using cell culture and RNA interference (RNAi) methods on the Human Glial Cell line MO3.13, the effects of psychosine accumulation in oligodendrites with correlation to GLD were determined(J.S. Won*et al.*, 2013).

It was observed that silencing of the GALC gene in MO3.13 cell line caused the accumulation of high levels of psychosine. Then, the various effects of psychosine on the differentiation and functioning of OLs were observed.

## 6.2. Effect of Psychosine on Oligodendrocyte life cycle

Very little difference was found regarding the change in cell morphology between GALC-silenced cells and the control cells. However, the cells with silenced GALC gene underwent severe cell body atrophy following initiation of OL differentiation. Moreover, these cells also underwent apoptotic cell death after three days following differentiation (J.S. Won *et al.*, 2013).

It was also found that psychosine induces cell death, at least in part, via the apoptotic pathway. The increased levels of psychosine have been found to increase the levels of the apoptosis marker Annexin V (J.S. Won, 2013).

## 6.3. Effect of Psychosine on Peroxisome Functioning

Peroxisomes play an essential role in the formation and maintenance of myelin by synthesizing myelin lipid components, such as plasmalogens, polyunsaturated fatty acids and cholesterol. In addition, peroxisomes are the major site for hydrogen peroxide production by oxidases and its degradation by catalase (J.S. Won, 2013).

In differentiating OLs, psychosine has been found to decrease the cellular expression of DHAP-AT, which is the first rate-limiting enzyme in the synthesis of plasmalogens. It also disturbs the mRNA expression of Peroxisome Proliferator-activated Receptor-α (PPARα) and Peroxisomal biogenesis factor 11 (PEX11),which are important factors in the division and proliferation of peroxisomes. In addition to these peroxisomal proteins, silencing of GALC in MO3.13 cells was also found to reduce proteolipid protein (PLP) gene expression, which suggests that perhaps psychosine accumulation during the OL differentiation may inhibit OL maturation process by inhibiting peroxisomal function as well as by reduced expression of myelin components (J.S. Won, 2013).

Hence, it can be concluded that the accumulation of psychosine in OLs plays an important role in inhibiting the proper differentiation of OLs in the brain, perhaps by inhibiting the functions of peroxisome (J.S. Won, 2013).

## 6.4. Effect of Psychosine on differentiation of OPCs to OLs

GALC-silenced oligodendrocyte precursor cells (OPCs) did not show any apparent change in cell morphology or decrease in metabolic processes initially. However, the mRNA expression of PPARα and DHAP-AT was markedly decreased in the GALC silenced differentiating OLs (J.S. Won, 2013).

Similarly, mRNA expression for MBP was also decreased. Along with the decreased mRNA expression of MBP, the OLs differentiated from OPCs silence for GALC had a much reduced number of dendritic processes as compared to OLs differentiated from control OPCs (J.S. Won, 2013).

Some of GALC-silenced cells undergoing differentiation also underwent cell death as observed by fragmented cell bodies. This indicates that psychosine accumulation in GALC-silenced OPC impedes OPC differentiation to mature OLs by inhibiting synthesis of myelin precursors (peroxisomal lipids and myelin proteins) as well as by decreasing cell viability (J.S. Won, 2013).

It was also found that increased levels of psychosine in OLs led to the production of various apoptotic factors and decreased the production of various OL-differentiating factors, such as proteolipid proteins (PLPs) (J.S. Won, 2013).

## 6.5. Effect of Psychosine on PKC activity

Protein kinase C (PKC) is an important signal transduction protein involved in regulating numerous cellular functions. Psychosine is a known inhibitor of PKC. Phosphorylated PKC (p-PKC, active) does not translocate to the plasma membrane of cells treated with psychosine under PKC-stimulating conditions.

In a group of experiments carried out using the enantiomer of psychosine, named *ent*-psychosine, it was observed that *ent*-psychosine still inhibited the translocation of PKC (J.A. Hawkins, 2013). This suggests that psychosine inhibition of PKC is most likely due to an alteration of the lipid environment of the plasma membrane rather than direct binding of psychosine to PKC itself. It is possible that psychosine may act by altering the plasma membrane in such a way as to exclude PKC association without binding to PKC.

## 6.6. Effect of Psychosine on Membranes

One possibility of psychosine activity and its contribution towards GLD pathogenesis is that it may have a global and physicochemical effect on the membrane environment and little or no direct binding to specific proteins. Indeed, several lines of evidence would seem to suggest a membrane-based toxicity mechanism.

Psychosine causes hemolysis, causes the inhibition of cytochrome c oxidase irrespective of the protein's orientation, and has never been shown to bind directly to any receptor or signaling protein despite significant investigation. It has also been noted to affect many proteins and cellular systems, which makes it extremely unlikely that it goes and interacts (or interferes) with each of them individually.

However, it has been observed that increased levels of psychosine leads to swelling in the membrane of liposomes. Disruptive lipids cause the liposomes to swell, whereas more inert lipids have no effect.

Psychosine is known to accumulate in detergent-resistant membrane (DRM) microdomains and to increase the percentage of cellular cholesterol there. Psychosine accumulates in cholesterol-rich microdomains and may have some effect on the protein composition of these domains, since it has been found to reduce the extractability of cholesterol from the membrane (J.A. Hawkins, 2013).

Hence, the possible impact of psychosine on cellular activities which may lead to GLD may be due to alteration of the membrane structures, including that of lysosomes, to which the disease is directly related to.

## 7. Disease Models for the study of GLD:

Since GLD is a disease with such a complex mechanism and such predominant neurological manifestations, and since the understanding of the disease is so limited, it is extremely important to study the disease for further understanding of its pathophysiology, its mode of progression and the various other factors related to it. However, the use of a human-based model is not feasible in a diseases like GLD due to various reasons, including ethics, high cost, etc.

Like most other neurodegenerative diseases, various disease models have been utilised for the study of GLD. In the conventional method, animal models are used for studying the physiological progression of the disease. The most popular animal model for the study of GLD has been the Twitcher mouse model. The recent trends, however, has been to use cell-based models, which are based on cell lines, such as MO3.13. The use of animal models are not preferred at all times due to their limitations at observing instantaneous as well as periodic changes at a biochemical and cellular level. Use of animals are also restricted due to ethical reasons.

### 7.1. Twitcher Mouse:

The Twitcher mouse was discovered in 1976 among the mouse colony maintained in the Jackson Laboratory, Bar Harbor, Maine.Its gene symbol was assigned as "*twi*" (K. Suzuki, 1983). The *twi* allele was later transferred from its original strain to a different strain to produce a new mutant which has been used to perform most of the experiments. This mouse model is specifically used for GLD because this mutated mouse actually suffers from the murine form of the disease. It shows alomost all the symptoms of GLD as seen in the human form, including the excessive accumulation of psychosine and the deficiency or defect of enzyme

galactocerebrosidase. Advantages include ease of maintenance and rapid reproduction of the mice. However, the use of this model is limited due to ethical concerns.

### 7.2. MO3.13 Cell Lines:

MO3.13 is a type of immortal human-human hybrid cell line. It has been found to express the typical phenotypic characteristics of primary oligodendrocytes. It was created by the fusion of a 6-thioguanine-resistant mutant of the human rhabdomyosarcoma RD (cancer of skeletal muscle) with adult human oligodendrocytes by a lectin-enhanced polyethylene glycol procedure. Unlike the tumor parent, MO3.13 has been shown to express surface immunoreactivity towards galactosyl cerebroside (GS) and intracellular immunoreactivity towards myelin basic protein (MBP), proteolipid protein (PLP), and glial fibrillary acidic protein (GFAP). MBP and PLP actions are affected due to presence of psychosine, the sphingolipid thought to be at the centre of GLD pathology. Upon differentiation, cells from this cell line have also been found to express MBP and MOG (myelin oligodendrocyte glycoprotein), which are proteins expressed by mature oligodendrocytes. Inducing mutations in the GALC gene can and has been used to study the impact of individual as well as various mutations altogether in GLD. This method is also being used to test various treatments for GLD.These factors and advantages make this cell line ideal for the study of GLD.

## 8. Treatment of GLD:

There is no known treatment to GLD. No drugs have been found which are effective against the accumulation of psychosine. Most of the available or tried treatments of GLDrevolve around stem-cell therapy, including hematopoietic stem cells, etc. This is perhaps due to the fact that much light needs to be shed on the working and various related factors of the disease. However, various other innovative methods are being theorised for the treatment and improving the condition of patients with GLD. Most of these methods of treatment are now being attempted and tried out on the murine model of the disease, using the twitchermice.

### 8.1. Bone marrow Transplantation (BMT):

It has been observed that enzyme-deficient oligodendrocytes and Schwann cells uptake the enzyme GALC. Hence, bone marrow transplantation from a congenic, normal mouse into a twitcher mouse have been attempted, due to the chances of macrophages migrating to the brain and secreting the enzyme (Y. Li*et al.*, 2014). However, this method has not proved to be of much effect as the amount of increase in GALC enzyme concentration in the CNS was found to be around 15%. And the life span was also extended pretty less. Hence, it may not serve as good method of treatment.

### 8.2. HSC Transplantation:

Another attempted method has been to transplant hematopoietic stem cells (HSC). These stem cells may be made to overexpress GALC and this overexpressed enzyme can be taken up by the enzyme-deficient cells, which has already been observed (Y. Li*et al.*, 2014). However, one of the roadblocks to this method has been the toxicity caused by the overexpression of this enzyme.

However, HSCs which have been differentiated into progenitor cells have not been found to show toxicity from overexpression of GALC, hence attempts have been made to induce overexpression in the differentiated progenitor cells.

## 8.3. Mesenchymal Stem Cells (MSCs):

Mesenchymal stem cells (MSCs) derived from the bone marrow and adipose tissues display the potential to differentiate into cells of adipose tissues, muscles and neuronal lineages. MSCs also have been found to produce and secrete GALC (Y. Li*et al.*, 2014). However, the effectiveness of MSCs is fairly low, even though its use has shown to reduce neuroinflammation.

## 8.4. Neuronal Stem Cells (NSCs):

Neuronal stem cells, which can be transduced to produce recombinant GALC by aviral vector-based model, have been theorised.Theoretically, transduction carried out using viral vector-based methods should induce the overexpression and subsequent secretion of GALC. This should allow the improvement in the condition of GALC-deficient cells by cellular uptake of GALC, while the NSCs themselves may perhaps differentiate and replace damaged neuronal cells (Y. Li*et al.*, 2014).

## 8.5. Enzyme Replacement Therapy (ERT):

A direct method targeting the deficient enzyme has been attempted by enzyme replacement therapy. Introduction of the enzyme galactocerebrosidase (GALC), produced using recombinant DNA technology, into the brain via peripheral administration or intracerebroventricular (ICV) administration has been found to reduce psychosine levels in the twitcher mouse model for GLD (W.C. Lee *et al.*, 2007). While peripheral administration has been found to effective in reducing levels of psychosine in the brain, along with rise in GALC activity, its activity has been limited to the periphery of the brain as the enzyme got accumulated along the periphery.

Another method that has been tested to introduce the enzyme into the brain is by intracerebroventricular administration of a single-dose of the enzyme. Recombinant GALC has been found in sites away from the point of injection 24 hours after injection. It leads to decrease in psychosine concentrations and increase in GALC activity. This method has also been found to increase the life span of the twitcher mice it was used on.

Hence, enzyme therapy can be applied as a novel method for the treatment of GLD even after the onset of the disease. One of its side effects may be high costs of treatment, high degree of skilled professionals and applying this method of treatment for the entire lifetime of the person.

## 8.6. Viral vector-mediated Gene Therapy:

Viral vectors hold extremely high potential as delivery mechanism of geneticmaterial such as DNA or cDNA, into various hosts. This method is currently in application for the production of recombinant strains for the production of various genetically-engineered products. This method holds high promise towards the treatment of various metabolism-related diseases, as the defective genes related to the affected metabolism can replaced by the proper genes, after they have been isolated,

by introducing these genes into the cells of affected individuals to repair these defects. This method holds much promise towards the treatment of GLD.

Vector-mediated gene therapy utilizes a replication-defective virus to deliver the GALC cDNA to GALC-deficient cells (Y. Li*et al.*, 2014). This method has been tried out on the twitcher mice models of GLD. Recombinant adeno-associated viruses (AAVs) have proved to be the most effective`e vectors for the delivery of the required genes to the affected cells in twitcher mouse.

When the required gene was delivered to the affected cells present in the cerebellar vermis, cortex, and hippocampus, the efficacy of the treatment and induction of GALC production was found to be higher and for a longer time period.Hence, this method may be of extreme importance for the treatment of this disease in the future.

### 8.7. Substrate Reduction Therapy (SRT):

Substrate reduction therapy (SRT) is based on the use of small compounds which inhibit the synthesis of psychosine.L-cycloserine is an irreversible inhibitor of 3-ketodyhydrosphingosine synthase, one of the enzymes required for psychosine synthesis (Y. Li*et al.*, 2014). Low-quantity dosagesprovided to rats has been found to decrease the levels of psychosine in affected cells.Repeated usage of this method has been found to be effective in reducing psychosine levels. However, as the usage of this method inhibits the production of other metabolites after psychosine synthesis in the related metabolism, it becomes a very narrow spectrum method. And its use will be limited to specific cases.

### 8.8. Mutiple combined therapies for GLD:

Even after all the various attempted methods, none has been found to be of high efficacy towards the treatment of GLD. The improvements caused by these therapies are rather insignificant for the proper treatment of this disease.Hence, among the various new approaches, one is to target the disease and its mechanism from various aspects. Hence, multiple combined therapies is being considered as one of the new strategies for the treatment of GLD(Y. Li*et al.*, 2014).Among the various treatments proposed by this method include SRT + BMT, anti-oxidant + anti-inflammatory therapy, ERT + BRT, viral vector-mediated gene therapy + BMT, etc.

## 9. Conclusion:

Globoid-cell leukodystrophy (GLD) is a neurodegenerative disorder which affects the myelin sheath of oligodendrocytes and Schwann cells of affected individual. Albeit rare, the impact of GLD is much intense on those affected. The onset of the disease is much early in the life of those affected, around the age of 6 months to 5 years. GLD leads to various neurological implications and those affected usually ultimately succumb to various unrelated infections.

GLD is caused by the deficiency in the GALC gene which codes for the enzyme galactocerebrosidase which is present in lysosomes. GALC degrades galactosphingolipids,with its primary substrate being galactosceerebroside. However, GLD is caused due to the excessive accumulation of the secondary substrate of GALC, psychosine. Psychosine accumulation in the oligodendrocytes leads to various effects such as improper functioning of peroxisomes, impact on NKCs, impact on the development and differentiation

of oligodendrocytes and various other proteins. It may also have a role in inducing apoptosis in some cells.

No particular treatment is known for GLD. But a variety of methods are being theorised, tried and tested on the various disease models of GLD, specifically the Twitcher mouse model. Most of the attempted treatments are based around stem cell therapy and transplantation, including hematopoietic stem cells (HSCs) transplantation. Various other methods have also been approached such as substrate reduction theory (SRT), gene therapy, enzyme replacement therapy (ERT) and various combined therapies. However, no presently applied method of treatment has made a proper impact on the disease.

GLD holds much fields of interest for further research. Much more needs to be known regarding various factors related to GLD, including its pathophysiology, the effects of psychosine on the various metabolic activities of oligodendrocytes, the mutations of GALC as well as the various methods of treatment of the disease. The understanding obtained from these future researches may one day pave the way for the proper treatment, and maybe even prevention, of this disease. Hence, one can hope that one day there might be a cure for GLD and the world may head towards a GLD-free era.

## References:


1. Knud Krabbe; ANew Familial Infantile Form of Diffuse Brain-Sclerosis; vol. 39, Pg.No. 74-114; 1916.

2. Je-Seong Won, Jinsu Kim, Manjeet Kaur Paintlia, Inderjit Singh, and Avtar K. Singh; Role of Endogenous Psychosine Accumulation in Oligodendrocytes Differentiation and Survival: Implication for Krabbe Disease; vol. 1508; Pg. No. 44–52; 2013.
3. Ludovico Cantuti Castelvetri, MariaI. Givogri, Amy Hebert, Benjamin Smith, Yuyu Song, Agnieszka Kaminska, Aurora Lopez-Rosas, Gerardo Morfini, Gustavo Pigino, Mark Sands, Scott T. Brady, and Ernesto R. Bongarzone; The Sphingolipid Psychosine Inhibits Fast Axonal Transport in Krabbe Disease by Activation of GSK3 and Deregulation of Molecular Motors; vol. 33(24); Pg. No. 10048–10056; 2013.
4. Jacqueline A. Hawkins-Salsbury, Archana R. Parameswar, Xuntian Jiang, Paul H. Schlesinger, Ernesto Bongarzone, Daniel S. Ory, Alexei V. Demchenko, and Mark S. Sands; Psychosine, the cytotoxic sphingolipid that accumulates in globoid cell leukodystrophy, alters membrane architecture; vol. 54; Pg. No. 3303-3311; 2013.
5. Yedda Li and Mark S. Sands; Experimental Therapies in the Murine Model of Globoid Cell Leukodystrophy; vol. 51(5); Pg. No. 600-606; 2014.
6. Chris H. Hill, Stephen C. Graham, Randy J. Read, and Janet E. Deane; Structural snapshots illustrate the catalytic cycle of β-galactocerebrosidase, the defective enzyme in Krabbe disease; vol. 110; Pg. No. 20479–20484; 2013.
7. Tatiana Suemi Sano; Krabbe disease: the importance of early diagnosis for prognosis; 10(2); Pg. No. 233-235; 2012.
8. Janet E. Deane, Stephen C. Graham, Nee Na Kim, Penelope E. Stein, Rosamund McNair, M. Begoña Cachón-González, Timothy M. Cox, and Randy J. Read; Insights into Krabbe disease from structures of galactocerebrosidase; vol. 108; Pg. No. 15169–15173; 2011.
9. Azzam A. Maghazachi; On The Role of Natural Killer Cells in Neurodegenerative Disease; vol. 5; Pg. No. 363-375; 2013.

10. Jameson J. Ribbens, Ann B. Moser, Walter C. Hubbard, Ernesto R. Bongarzone, and Gustavo H.B. Maegawa; Characterization and application of a disease-cell model for a neurodegenerative lysosomal disease; vol. 111(2); Pg. No. 172–183; 2014.
11. Wing C. Lee, Yuen K. Tsoi, Frederick J. Troendle, Michael W. DeLucia, Zeshan Ahmed, Chad A. Dicky, Dennis W. Dickson, and Christopher B. Eckman; Single-dose intracerebroventricular administration of galactocerebrosidase improves survival in a mouse model of globoid cell leukodystrophy; vol. 21; Pg. No. 2520-2527; 2007.
12. Ludovico Cantuti-Castelvetri, ErickMaravilla, MichaelMarshall, TammyTamayo, Ludovic D'auria, John Monge, James Jeffries, Tuba Sural-Fehr, AuroraLopez-Rosas, GuannanLi, Kelly Garcia, Richard van Breeman, Charles Vite, Jesus Garcia and ErnestoR.Bongarzone;MechanismofNeuromuscularDysfunctioninKrabbe Disease; vol. 35(4); Pg. No. 1606–1616; 2015.
13. Kunihiko Suzuki, MD, and Kinuko Suzuki, MD; The Twitcher Mouse : A Model of Human Globoid Cell Leukodystrophy; vol. 111; Pg. No. 394 – 397; 1983.
14. http://www.ncbi.nlm.nih.gov/gene/2581