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Mechanisms of demyelination and neurodegeneration in globoid cell leukodystrophy

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Abstract

Globoid cell leukodystrophy (GLD), also known as Krabbe disease, is a lysosomal storage disorder causing extensive demyelination in the central and peripheral nervous systems. GLD is caused by loss-of-function mutations in the lysosomal hydrolase, galactosylceramidase (GALC), which catabolizes the myelin sphingolipid galactosylceramide. The pathophysiology of GLD is complex and reflects the expression of GALC in a number of glial and neural cell types in both the central and peripheral nervous systems (CNS and PNS), as well as leukocytes and kidney in the periphery. Over the years, GLD has garnered a wide range of scientific and medical interests, especially as a model system to study gene therapy and novel preclinical therapeutic approaches to treat the spontaneous murine model for GLD. Here, we review recent findings in the field of Krabbe disease, with particular emphasis on novel aspects of GALC physiology, GLD pathophysiology, and therapeutic strategies.

Keywords

demyelination; Krabbe disease; leukodystrophy; neurodegeneration

1 | INTRODUCTION

Myelination is an evolutionary adaptation of vertebrates, which facilitates saltatory electrical conduction along axons and allows for more rapid neural transmission (Tasaki, 1939). Myelin exists in both the central and peripheral nervous systems (CNS and PNS, respectively) and is formed by the wrapping of oligodendrocyte or Schwann cell plasma membrane around axons. This process is exquisitely regulated and many different

genetic defects can result in a hypomyelinating leukodystrophy (reviewed in Wolf, Ffrench-Constant, & van der Knaap, 2021). Compared to other membranes, myelin has a very high lipid content and an unusual composition, specifically enriched in glycosphingolipids (galactosylceramide and sulfatide; Chrast, Saher, Nave, & Verheijen, 2011). Myelin is an incredibly stable structure (M. E. Smith, 1968), likely influenced by its unique lipid and protein composition. In fact, evidence of intact myelin sheath with still detectable galactosylceramide was present in nerve specimens from remnants of a 5,000 year old ancient hominoid (Hess et al., 1998). Despite its stability, myelin is continuously remodeled to some degree postdevelopment (Lasiene, Matsui, Sawa, Wong, & Horner, 2009; Yeung et al., 2014; Young et al., 2013) and in response to injury and disease. The process of myelin degradation is complex and relies on a number of proteins and mechanisms in both myelinating cells and cells that do not make myelin. Of known importance for myelin turnover is the presence of the glycosphingolipid specific hydrolyzing enzyme, galactosylceramidase (GALC).

Globoid cell leukodystrophy (GLD, also Krabbe disease, “KD”) was first described by the Danish neurologist Knud Krabbe (Krabbe, 1916). GLD is caused by mutations in the *GALC* gene, that encodes a lysosomal enzyme catabolizing the myelin lipid component galactosylceramide (GalCer) (Figure 1). GALC deficiency causes rapid and severe demyelination, neurodegeneration, and neuroinflammation of both the CNS and PNS (Figures 2 and 3). The incidence of GLD is estimated at 1:100,000–1:250,000 (Barczykowski, Foss, Duffner, Yan, & Carter, 2012) and is classified either as infantile-onset (presentation within the first year of life, mean survival 24 months) or late-onset (presentation after the first year of life, mean survival 4–6 years after onset; Orsini, Escolar, Wasserstein, & Caggana, 1993).

The human *GALC* gene is mapped to chromosome 14q13 (Cannizzaro, Chen, Rafi, & Wenger, 1994) and more than 130 mutations have thus far been catalogued in the Human Gene Mutation Database (HGMD). At least 128 of those mutations have been reported to cause GLD (<http://www.hgmd.org>; Wenger, Rafi, Luzi, Datto, & Costantino-Ceccarini, 2000). The most common mutation in the European patient population is a large deletion spanning exons 11–17 (Rafi, Luzi, Chen, & Wenger, 1995), c.1161+6532_polyA+9kdel (IVS10del30kb) that is always present with a c.550C>T (502C>T) polymorphism on the same allele, which eliminates the entire coding region for the 30-kDa enzyme subunit and about 15% of the coding region for the 50-kDa subunit. This mutation is absent in the Japanese population, which is another large GLD cohort. Instead, Japanese patients have a 12 bp deletion along with a 3 bp new insertion either c.683_694del12insCTC or c.2002A>C (635_646del12insCTC or 1954A>C; C. Xu, Sakai, Taniike, Inui, & Ozono, 2006), resulting in deletion of 5 amino acids and insertion of 2 amino acids that affects the formation of GALC quaternary structure (Tatsumi et al., 1995; Wenger, Rafi, & Luzi, 1997). In addition, a high prevalence of the infantile form was detected in two separate inbred communities in Israel. The Druze population commonly have a c.1796T>G (1748T>G) transversion in exon 15, while the Moslem Arab population have a c.1630G>A (1582G>A) transition in exon 14 (Rafi, Luzi et al., 1996).

The infantile form of GLD accounts for almost 90% of the total cases of the patients (Orsini et al., 1993). Clinical features of the classical infantile form of GLD include progressive neurologic deterioration with decorticate posturing, peripheral neuropathy, blindness, deafness, autonomic instability, and death (Orsini et al., 1993). Initial symptoms of untreated children include irritability, feeding difficulties and poor head control. As the disease progresses, affected children experience loss of vision along with hyperactive reflexes and deterioration of motor and cognitive function (Suzuki, 2003). Children with GLD and/or other leukodystrophies are usually also prone to respiratory and urinary tract infections (Anderson et al., 2014) as well as a host of secondary complications. The most advanced stages of infantile GLD are very severe and progress to paralysis and severe neurologic decline (Escobar, West, Dallavecchia, Poe, & LaPoint, 2016).

While supportive care significantly improves the quality of life for GLD patients, hematopoietic stem cell therapy (HSCT) is the only approved disease-modifying treatment for GLD, and only asymptomatic patients benefit from this procedure.

Despite the relatively low incidence of KD, an extensive number of research studies have explored disease pathogenesis and therapeutic interventions in KD. This was facilitated by the long-term availability of spontaneously occurring disease models of KD among various species including dogs (Fletcher, Suzuki, & Martin, 1977; Hirth & Nielsen, 1967; Victoria, Rafi, & Wenger, 1996), cats (Johnson, 1970; Sigurdson, Basaraba, Mazzaferro, & Gould, 2002), sheep (Pritchard, Naphthine, & Sinclair, 1980), and primates (Baskin et al., 1998; Luzi, Rafi, Victoria, Baskin, & Wenger, 1997). In particular, the *twitcher* mouse model, first characterized in 1980 by the Jackson laboratory, was one of the earliest leukodystrophy and LSD models discovered in mice (Duchen, Eicher, Jacobs, Scaravilli, & Teixeira, 1980). Accordingly, there have been nearly 100 studies using the *twitcher* mouse model, ranging in focuses from disease mechanism, gene therapy (GT), stem cell transplantation, and remyelination strategies. Despite the abundance of research, a number of underlying mechanistic questions remain unanswered, including the normal biology and function of GALC and the pathogenesis of demyelination resulting from its absence. Here, we review the current understanding of GALC, GLD pathogenesis, and therapeutic strategies, focusing on recent work that has provided a new understanding of this process.

2 | PHYSIOLOGIC ROLE OF GALC

2.1 | Galactosylceramide and myelin

The major physiologic substrate for GALC is the sphingolipid galactosylceramide (GalCer). Sphingolipids are a group of bioactive lipids that share a common sphingosine backbone. Sphingolipids act both as structural components and signaling molecules regulating cell death, proliferation, and cell differentiation. The structural makeup of sphingolipids includes a sphingolipid backbone, which can be modified with a fatty acid group to form ceramide (reviewed in Gault, Obeid, & Hannun, 2010). The two major pathways of sphingolipid metabolism include a de novo synthesis pathway, in which serine, palmitoyl-coA, and stearoyl-coA are enzymatically modified to generate ceramides. Alternatively, a sophisticated salvage pathway exists, in which sphingolipids can be extensively modified to form a number of different species with unique functions. Within the salvage pathway, there

are six different endoplasmic reticulum (ER) enzymes that exist for ceramide synthesis, CerS 1–6, each of which generates ceramides of unique fatty acid lengths (Gault et al., 2010). The length of the fatty acid component dictates the biophysical properties and hydrophobicity of the sphingolipid. For example, myelin ceramides tend to be of very long fatty acid length (C22–C24) and are generated primarily by CerS2 (Imgrund et al., 2009). Ceramides are subsequently modified with various polar head groups including glucose or galactose moieties in variable sequences (gangliosides, globosides, and glycosphingolipids) or phosphocholine (sphingomyelin). Both sphingosine and ceramide can also be phosphorylated, to form sphingosine-1-phosphate (S1P) and ceramide-1-phosphate, important mediators of cell survival and death. Interestingly, a number of S1P analogues are popular pharmacologic agents for demyelinating diseases like Multiple Sclerosis, though their mechanism is thought to work primarily through immune modulation (Brinkmann et al., 2010). Notably, a recent article reports that S1P receptor agonist improves demyelination in *twitcher* mice (Béchet, O’Sullivan, Yssel, Fagan, & Dev, 2020).

GalCer is a sphingolipid component of the outer leaflet of plasma membranes in many different cell types (Blanchette, Lin, Ratto, & Longo, 2006) and is synthesized by the galactosylation of ceramide by an ER enzyme, ceramide galactosyl transferase (CGT, [Sprong et al., 1998]). Like most sphingolipids, both hydroxylated and non-hydroxylated forms of GalCer exist, though their specific functions are not well understood. While GalCer may be expressed by many cell types, it is especially abundant in the nervous system where it represents 2% of grey matter and 12% of white matter, hence its historic terminology as “cerebroside” or “galactocerebroside” dating to 1874 (Fry, Weissbarth, Lehrer, & Bornstein, 1974; Thudichum, 1874). The abundance of GalCer in brain white matter is due to its enriched localization in myelin sheaths, created by oligodendrocyte membranes (or Schwann cells in the peripheral nervous system; Figure 1). Indeed, GalCer reaches up to 24% of total lipids in myelin sheaths (Garbay, Heape, Sargueil, & Cassagne, 2000; Norton & Cammer, 1984; O’Brien, 1965). In fact, the popular oligodendrocyte marker “O1” recognizes a GalCer antigen (Bansal, Warrington, Gard, Ranscht, & Pfeiffer, 1989; Sommer & Schachner, 1981). GalCer also serves as a substrate for synthesis of the sphingolipid sulfatide, which is another myelin-enriched lipid component (recognized by the “O4” oligodendrocyte marker). Of note, the myelin predominant species of both GalCer and sulfatide contain very long fatty acyl chains comprised of 22–24 carbons. This is in contrast to the composition of most cellular sphingolipids, which often span 16–18 carbons in length.

It is hypothesized that the function of myelin GalCer and sulfatide aid in membrane compaction and stability. These galactosphingolipids are localized in the extracellular surfaces of myelin, forming the intraperiod line in the wrapped myelin sheath (Boggs, Gao, & Hirahara, 2008; Figure 1). Knockout animals for CGT, which were unable to generate GalCer or sulfatide, had only minor defects in both CNS and PNS myelin formation and compaction, but a clinical phenotype possibly due to altered neurophysiological properties of myelin and defects in myelin maintenance (splitting; Bosio, Binczek, & Stoffel, 1996; Coetzee et al., 1996). The altered neurophysiological properties of CGT-null myelin are likely due to disruption of axonal paranodal domains (Dupree, Girault, & Popko, 1999). Interestingly, myelin of the CGT-null tissues was paradoxically enriched for glucosylceramide (GluCer), suggesting a compensatory presence of this lipid for myelin

compaction, but not for myelin insulating function. Surprisingly, further investigation revealed an overall minor role of GluCer in myelin function. Independent oligodendrocyte deletion of UDP-glucose ceramide glucosyltransferase (Ugcg), thereby preventing the synthesis of glucosylceramide, did not cause a myelin defect (Saadat et al., 2010). Similarly, compound deletion of oligodendrocyte UGCG and global CGT strongly resembled the global CGT-null myelin, suggesting that the function of myelin galactosphingolipids is important and distinct from glucosphingolipids (Saadat et al., 2010). Interestingly, sulfatides, rather than GalCer, seems to be crucial for formation of paranodal domains and to prevent myelin splitting. Mice with genetic deletion of galactosyl 3'-sulfotransferase (CST), which prevents the generation of sulfatide while permitting the generation of GalCer, had similar paranodal anomalies (Honke et al., 2002). This highlights the unique functional roles in myelin of galactosylceramide and sulfatides from other sphingolipids and emphasizes the necessity for further investigation regarding myelin sphingolipid structure and function.

2.2 | Lysosomal GALC

The myelin sphingolipid substrate GalCer is degraded by the lysosomal hydrolase GALC. Lysosomes are membrane bound organelles that serve as the major site of macromolecule catabolism in the cell. There are currently over 200 lysosomal proteins identified (Pu, Guardia, Keren-Kaplan, & Bonifacino, 2016), categorized generally as acid hydrolases involved in specific catabolic processes or lysosome membrane proteins involved in lysosome stability and regulation. Lysosomes are maintained at a pH of 4.5–5 by an ATP-dependent proton pump and contain >50 different hydrolytic enzymes (hydrolases) that catabolize a large range of macromolecules including glycosides, sulfates, phosphates, lipids, phospholipids, proteins, and nucleic acids.

The deficiency of a single lysosomal hydrolase will result in the inability to degrade the enzyme's respective substrate, resulting in a specific lysosomal storage disorder (LSD; reviewed in Ferreira & Gahl, 2017). Nearly one third of the lysosomal hydrolases are involved in lipid metabolism (Lübke, Lobel, & Sleat, 2009), nine of which specifically degrade sphingolipids. The sphingolipid polar head groups are cleaved by one of eight different hydrolases, while acid ceramidase cleaves the final ceramide backbone into sphingosine. Additionally, there are multiple sphingolipid hydrolase coactivators that facilitate sphingolipid catabolism. Of particular interest are the prosaposin derived activators, saposins A–D, which are transcribed and cleaved from a common precursor. Each saposin seems to have the ability to activate the function of a specific sphingolipid hydrolase, emphasizing the nonoverlapping nature of these enzymes (Schuette, Pierstorff, Huettler, & Sandhoff, 2001).

Precursor GALC is synthesized in the ER as a 80-kDa precursor protein with three structural domains. Like many lysosomal hydrolyzing enzymes, it is cleaved in the lysosome into two subunits, a 50- and a 30-kDa subunit. The functional significance for the cleavage of GALC remains unknown. In vitro data suggests that the precursor form may be enzymatically active, though the pH requirements for GALC function imply that most GALC activity likely occur in the lysosome, where the cleaved forms predominate (reviewed in Shin, Feltri, & Wrabetz, 2016). Like other sphingolipid enzymes, GALC works in concert with one of

the four saposins, specifically saposin A (SapA), thought to help shuttle the sphingolipid to the enzymatic active site. Recent crystal structure data revealed that GALC functions as a compound heterodimer comprised of two units of GALC and two units of SapA (Hill et al., 2018). Genetic knockout mice of SapA showed similar (albeit delayed) pathology to GALC knockout animals (Matsuda et al., 2001), emphasizing the congruent functions. A small number of patients with clinical and pathological features resembling GLD have been identified which include mutations in the SapA domain of PSAP rather than GALC (Spiegel et al., 2005).

2.3 | Trafficking of GALC and cross-correction

Lysosomal hydrolases are modified in the trans Golgi network with mannose-6-phosphate (M6P) residues via GlcNAc phosphotransferase. M6P-tagged hydrolases are then properly sorted for lysosomal delivery via M6P receptor (MPR) binding in the Golgi (Coutinho, Prata, & Alves, 2012). In the low pH of the lysosome, the hydrolase-MPR complex dissociates. Hydrolases remain in the lysosomal matrix while unbound MPR is recycled to the Golgi apparatus. Some of the synthesized M6P-tagged enzymes is thought to escape the lysosome targeting and are secreted in the extracellular space where they can be taken up by cells using the same MPR, which are found (at reduced levels) on the plasma membrane. This process thereby provides a mechanism, termed cross-correction, in which neighboring cells can share lysosomal enzyme and is central to the rationale for many LSD therapies.

While cross-correction of lysosomal enzyme is an important concept in the LSD field, the extent of its role in biology and therapy remains to be precisely defined. Most studies have focused on in vitro characterization of this process (Fratantoni, Hall, & Neufeld, 1969; Neufeld & Fratantoni, 1970), with an emphasis on supplying lysosomal enzyme to neighboring deficient cells. However, some evidence has emerged questioning the efficiency of cross-correction in vivo in GLD, Metachromatic leukodystrophy and other neurodegenerative LSDs (Y. Kondo, Wenger, Gallo, & Duncan, 2005; Matthes et al., 2015). More recent experiments using in vitro and in vivo GALC knockout strategies showed that GALC negative or mutant cells are not able to uptake GALC through the M6P receptor pathway as efficiently as wild type cells (Weinstock et al., 2020). Furthermore, the overall biologic significance of GALC cross-correction in physiologic conditions remains a mystery. A recent conditional mouse was specifically engineered to produce a Cre-dependent, LAMP1-GALC fusion protein that maintained GALC activity while being tethered in the membrane of lysosomes, thus preventing secretion and cross-correction (Mikulka et al., 2020). Interestingly, *twitcher* mice expressing the LAMP1-GALC fusion protein did not have any symptoms and survived normally. This suggests that there is no apparent function for GALC secretion in normal physiology. Therefore, the role of cross-correction in therapy and biology for GALC and other lysosomal hydrolases is a major question for the field moving forward.

2.4 | Expression of GALC

The cellular expression of GALC seems to reflect the localization of its substrate GalCer, as it is expressed ubiquitously by all cell types of the nervous system though enriched in oligodendrocytes (Zhang et al., 2014). Interestingly, a growing body of evidence is emerging

regarding the expression of GALC in nonmyelinating cells. For example, GALC is highly expressed by neurons in a number of studies (Dolcetta et al., 2004; Weinstock et al., 2020; Zhang et al., 2014). Similarly, recent data indicates that macrophages, which normally produce low levels of GALC, dramatically upregulate GALC expression when recruited to sites of demyelination (Weinstock, Shin, et al., 2020). This raises the possibility that GALC gene expression can be dynamically regulated when lysosomes are challenged by the accumulation of its substrate. GALC protein is also expressed in astrocytes and microglia (Weinstock, Kreher, et al., 2020), and in several non-nervous system tissues such as kidney.

In addition to variability in the cellular expression of GALC, the temporal expression of GALC is also of interest. Previous studies hinted that GALC rescue experiments in GALC deficient mice and humans were most efficacious when delivered as early as possible (Rafi, Rao, Luzi, Curtis, & Wenger, 2012). While early treatment may seem intuitive, the precise temporal requirements for GALC were not previously recognized. A recent study found that GALC expression peaks in murine brain tissues shortly after birth, approximately 4–6 days postnatally. Intriguingly, temporal deletion of GALC at or before this period correlated with the development of more severe disease and suggests a precise mechanism governing GALC expression in early development (Weinstock, Kreher, et al., 2020).

The transcriptional regulation of GALC has not yet been elucidated, but transgenic mouse studies using a BAC expressing the human *GALC* gene and its upstream regulatory sequence, was sufficient to prevent GLD pathology in *twitcher* mice (De Gasperi et al., 2004). More recently, a landmark study showed that a master lysosomal transcription factor, TFEB, recognizes CLEAR elements and is present in this promoter region of *GALC* (Sardiello et al., 2009). By this theory, lysosomal enzymes are co-transcribed and should be co-expressed among all cell types. Data regarding more selective mechanisms of expression of unique lysosomal enzymes is limited. Nonetheless, a strong body of work surrounding TFEB has now been characterized regarding lysosomal biogenesis in response to amino acid metabolism. For example, when amino acid pools in the lysosome are low, the master nutritional sensor mTOR, which docks at the lysosomal membrane, releases inhibition of TFEB to orchestrate signaling cascades that induce autophagy and lysosomal biogenesis (Settembre et al., 2012). This work contributed to a paradigm shift to redefine the lysosome from a “dead-end” metabolic organelle, to a dynamic metabolic signaling hub for the regulation of the cell function. Whether a similar homeostatic process occurs in response to sphingolipids and GalCer metabolism remains to be determined.

3 | PATHOPHYSIOLOGY OF GLD

3.1 | Psychosine toxicity

As GALC is a lysosomal enzyme, GLD belongs to the superfamily of LSDs, characterized by widespread and severe tissue damage, engorgement and malfunction of lysosomes. However, unlike other LSDs, the primary GALC substrate, GalCer, does not broadly accumulate in patient tissues, and paradoxically even decreases (Vanier & Svennerholm, 1974). While this change may reflect overall myelin loss, in which GalCer is highly concentrated, the biochemical explanation for why GalCer does not accumulate relative to other lipids has been a major question in the field since the initial characterization

of KD. Instead, a minor substrate, psychosine, also known as galactosylsphingosine, accumulates. Psychosine is categorized as a lyso-sphingolipid, meaning it appears like a typical sphingolipid, in this case galactosylceramide, minus its acyl chain (Figure 2).

Psychosine is almost undetectable in normal tissues, is a potent toxin and is considered a major culprit for demyelination, loss of oligodendrocytes, and neuron degeneration in KD (“Psychosine hypothesis”; Miyatake & Suzuki, 1972). Psychosine is a known inducer of in vitro cell death (Tohyama, Matsuda, & Suzuki, 2001) in different cell types (Haq, Giri, Singh, & Singh, 2003; Jatana, Giri, & Singh, 2002; Tanaka & Webster, 1993), including those which express GALC (Formichi et al., 2007). Although the cytotoxicity of psychosine, in vitro, is well established, the actual mechanism has been difficult to elucidate and may be multifaceted. Descriptions of psychosine toxicity include inhibition of Protein Kinase C (PKC; Hannun & Bell, 1987, 1989), mitochondrial Cytochrome C oxidase (Igisu, Hamasaki, Ito, & Ou, 1988; Igisu & Nakamura, 1986; Ribbens, Moser, Hubbard, Bongarzone, & Maegawa, 2014), and caspase activation (Zaka & Wenger, 2004). Furthermore, psychosine has been shown to induce dispersion of subcellular organelles like the trans Golgi network and endosomal vesicles (Kanazawa, Takematsu, Yamamoto, Yamamoto, & Kozutsumi, 2008) and cause defects in peroxisomal (Khan, Haq, Giri, Singh, & Singh, 2005) and mitochondrial (Voccoli, Tonazzini, Signore, Caleo, & Cecchini, 2014) function. It was speculated that psychosine may exert its toxicity through a plasma membrane receptor, TDAG8, that would function as a “psychosine receptor” (Im, Heise, Nguyen, O’Dowd, & Lynch, 2001). However, a study using the enantiomer of psychosine, which in theory should prevent specific protein interactions, still had high toxicity (Hawkins-Salsbury et al., 2013). Recently, psychosine was proposed to alter the pH and degradative capacity of lysosomes, thus inducing broad lysosomal dysfunction (Folts, Scott-Hewitt, Pröschel, Mayer-Pröschel, & Noble, 2016).

Currently, the most widely accepted theory of psychosine toxicity is that psychosine (and other lysosphingolipids) can act as a detergent and disturb membrane microdomain organization of lipid rafts, thereby causing cell death (Hawkins-Salsbury et al., 2013; Spassieva & Bieberich, 2016; White et al., 2009). Psychosine can accumulate in lipid rafts of myelinating glia in brains and nerves very early in the disease process (White et al., 2009). In fact, psychosine can and does accumulate in a wide range of cell types in KD mice including blood cells and thymus cells (Chuang et al., 2013; Galbiati et al., 2007; Zhu, Lopez-Rosas, Qiu, Van Breemen, & Bongarzone, 2012). An emerging body of work also suggests that psychosine can accumulate in lipid raft organization of neurons and directly induce neuronal apoptosis (Castelvetri et al., 2011; Teixeira et al., 2014) or dysfunction (Castelvetri et al., 2011; White et al., 2009; White et al., 2011), and axonal degeneration due to abnormal neurofilament phosphorylation (Cantuti-Castelvetri et al., 2012), fast axonal transport (Cantuti Castelvetri et al., 2013), and neuromuscular junction function (Cantuti-Castelvetri et al., 2015). Psychosine has even been proposed to cause α -synuclein formation and neurodegeneration in Krabbe mice and patients (B. R. Smith et al., 2014). Therefore, the proposed mechanisms of psychosine toxicity are broad and much question still remains regarding the direct mechanism of psychosine-induced pathogenesis in KD.

3.2 | The molecular origin of psychosine

Even more controversial than the biological effect of psychosine in KD has been the biochemical and cellular origin of psychosine synthesis. Although a catabolic pathway from the direct deacylation of GalCer was logical, postulated decades ago, and repeatedly investigated, no biochemical evidence to support formation of psychosine via GalCer deacylation were achieved (reviewed in Suzuki, 2016). This led to the belief that psychosine is synthesized anabolically through the addition of galactose to sphingosine (Cleland & Kennedy, 1960; Y. N. Lin & Radin, 1973). However, the question remained of why the undigested GalCer did not accumulate, except possibly in crystals found abundantly in myelinating cells and globoid cells throughout the nervous system (Duchen et al., 1980; Irino & Suzuki, 1990; Jacobs, Scaravilli, & De Aranda, 1982; Okeda, Suzuki, Horiguchi, & Fujii, 1979; Takahashi, Igisu, Suzuki, & Suzuki, 1983; Yajima, Fletcher, & Suzuki, 1977).

In 2019, genetic experiments in the laboratory of Dr. Mark Sands solved this mystery. Genetic deletion of the catabolic enzyme acid ceramidase (*Asah1*) in twitcher mice completely abolished psychosine production, proving that psychosine is generated by the deacylation of GalCer by acid ceramidase (Li et al., 2019). This study was also significant for showing the direct toxic effects of psychosine, *in vivo*, and the extent of its contribution to pathology. The implication from this study is that GalCer does not accumulate significantly in KD because it is readily degraded into psychosine. Furthermore, this biochemical discovery has potential for significant advances for patients with KD, especially if an acid ceramidase inhibitor aimed at preventing psychosine formation proves efficacious. One note of caution for inhibiting acid ceramidase for therapeutic purpose is that acid ceramidase deficiency causes Farber Disease, a severe LSD characterized by accumulation of ceramide. The phenotype of compound twitcher/Farber mice was similar to that of Farber mice alone and less severe than twitcher alone. These compound mice had longer life spans compared to twitcher mice, as well as reduced demyelination and fewer globoid cells, ultimately proving the “psychosine hypothesis”: psychosine is indeed responsible for much of the demyelination and axonal degeneration present in KD. However, given the confounding effect of acid ceramidase deficiency that caused a Farber Disease phenotype in the mice, it was not possible to determine if psychosine is sufficient to explain the entirety of twitcher pathology. In fact, several lines of evidence suggest that psychosine, even if clearly toxic and an important cause of pathology, is not sufficient to cause the entirety of the KD phenotype. For example, *twitcher* mice completely deficient for the synthetic GalCer enzyme (CGT) were unable to produce psychosine but instead had a neurodegenerative phenotype (Ezoe et al., 2000a). Alternatively, mice replicating the human KD mutation *GALC* E130K, termed *twi-5J*, had less psychosine than traditional *twitcher* mice, but had a more severe phenotype (Potter et al., 2013).

A second major historic question in the field surround the cellular origin of psychosine production. Studying *twitcher* mice for over 40 years has yielded many important tenants of KD pathology including characterization of the severe demyelination occurring in the CNS and PNS, the prominent role of neurodegeneration and axonal pathology and the contribution of globoid cells to neuroinflammation (Duchen et al., 1980). However, given the ubiquitous nature of the W339X *Galc* nonsense mutation in *twitcher*, question

regarding cellular autonomy and the cellular origin of psychosine were challenging. The use of conditional mutagenesis in knock-out mice have been used for almost three decades (Gu, Zou, & Rajewsky, 1993) to elucidate cell autonomy in development and in many genetic diseases. However, the application of Cre-Lox technology to lysosomal storage diseases has generally been limited, due to the suspicion that cross-correction of GALC by nonrecombined cells would mask any phenotype caused by cell-specific GALC deletion.

Recently, two groups have generated independent approaches to floxed *Galc* animals to further pursue the question of cellular autonomy in KD pathogenesis. The first approach engineered a “non cross-correctable” Cre-dependent, LAMP1-GALC fusion protein expressed artificially by the Rosa26-promoter (Mikulka et al., 2020). Fusion GALC in these mice is tethered to the lysosomal membrane protein LAMP1. The second group followed a more traditional approach and engineered a GALC floxed allele allowing endogenous GALC expression, secretion, and thus cross-correction (Weinstock, Kreher, et al., 2020; Weinstock, Shin, et al., 2020). In both studies, *Galc* floxed animals were crossed to *Mpz-Cre*, which express Cre specifically in Schwann cells. The data generated from both studies showed definitively that Schwann cells produce psychosine autonomously, which is sufficient to generate a severe demyelinating neuropathy. The similarity of phenotypes of these two mutant mice, one impeding and one retaining the possibility of cross-correction, strongly suggests that cross-correction of GALC by unrecombined cells does not occur in vivo (Weinstock, Shin, et al., 2020). Further experiments in vitro showed that one reason for poor cross-correction is that GALC knockout or mutant cells are not able to uptake GALC through the M6P receptor pathway as efficiently as wild type cells. Importantly, despite equal amount of psychosine, the amount of demyelination and axonal degeneration was more severe in the global knockout than in the Schwann cell specific knockout, further indicating that psychosine does not account for the entirety of GLD pathology, and also that cells other than Schwann cells need GALC and contribute to overall disease.

3.3 | Macrophages and globoid reaction

GLD was originally diagnosed pathologically by the presence of pathognomonic Periodic Acid-Schiff (PAS) stained “globoid cells” in brains of autopsy patients (Figure 3). These multinucleated cells have a traditional storage phenotype including distended cytoplasm, engorged lysosomes, unique lipid crystals (presumably GalCer) (Austin, 1963; Jacobs et al., 1982; Yunis & Lee, 1970) and partially digested myelin sheaths. Globoid cells accumulate in areas of highest demyelination in KD tissues (Figure 3; D’Agostino, Sayre, & Hayles, 1963), and these storage-laden macrophages follow the spatio-temporal pattern of normal myelination (Taniike & Suzuki, 1994). There has been some disagreement as to whether globoid cells comes from infiltrating monocyte-derived macrophages or innate microglia (Nicaise, Bongarzone, & Crocker, 2016). Frequent observations of PAS-positive globoid cells and macrophages surrounding blood vessels in KD brains suggested that globoid cells represent infiltrating monocyte-derived macrophages from the periphery. Similarly, there has been some question as to whether macrophages can be converted to globoid cells via galactosylceramide (Austin & Lehfeldt, 1965) or psychosine. In addition to questions about the mechanistic origin of globoid cells, a larger question regarding their role in pathogenesis and the significance in disease progression remained unanswered and largely speculative.

The recently generated floxed *Galc* mouse was able to shed light on some of these important questions. The most striking difference between nerves from global and Schwann cell conditional knockout mice was the presence of globoid cells in the former but not the latter, pointing to macrophages and innate immune cells as independently requiring GALC (Weinstock, Shin, et al., 2020). Indeed, in vivo and in vitro experiments confirmed that macrophages require GALC to assist in phagocytosis and digestion of myelin. Specifically, when GALC deficient macrophages are confronted with GalCer (as opposed to psychosine), they transform into globoid cells and express a molecular phenotype associated with impaired phagocytosis, oxidative stress and a pro-inflammatory phenotype. Furthermore, the presence of globoid cells seems to negatively impact the overall health of the nerve and contributes to additional inflammation, demyelination and axonal degeneration.

Interestingly, the well-documented benefit of HSCT in presymptomatic KD patients seems to also exert its effect by providing the nervous system with phagocytic competent cells, rather than by cross-correction of GALC (Weinstock, Shin, et al., 2020). Analogous conclusions were recently obtained in a postmortem analysis of brains of patients with metachromatic leukodystrophy, a lysosomal storage disease due loss of function of Arylsulfatase A (Wolf et al., 2020). These data are also in line with previous studies that showed that GALC haplo-insufficient mice had mild defects in remyelination following cuprizone-induced demyelination (Scott-Hewitt et al., 2017; Scott-Hewitt, Folts, & Noble, 2018). The association of GALC-deficiency and associated myelin-induced inflammation is particularly fascinating as GALC was recently discovered to be a possible risk factor in patients with Multiple Sclerosis (Sawcer et al., 2011).

The direct ability for GalCer to produce toxicity is also particularly interesting because the same GalCer inclusions found in globoid cells are also present in Schwann cells and oligodendrocytes. Therefore, GalCer may contribute to disease pathology via its redistribution from normal myelin to abnormal crystal inclusions. A number of recent studies have shown that crystal accumulation in cells can be toxic due to the formation of the NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3) inflammasome (Duewell et al., 2010; Rajamäki et al., 2010) or lysosome membrane permeabilization (LMP; Ono et al., 2018). Various types of crystals have been documented to converge in a similar pathological mechanism in a wide range of pathologies affecting the innate immune system (in particular macrophages) including gout (Martinon, Pétrilli, Mayor, Tardivel, & Tschopp, 2006), asbestos and silica lung disease (Dostert et al., 2008), atherosclerosis (Duewell et al., 2010), and even noncrystal molecules like amyloid-beta in Alzheimer's disease (Halle et al., 2008). It should also be noted that myelin derived crystals were recently found to induce pathology in a demyelinating mouse model, though these crystals were proposed to be comprised of cholesterol (Cantuti-Castelvetri et al., 2018). The role of GalCer or crystal toxicity may, therefore, contribute to GLD pathology though much remains unknown.

3.4 | Neurodegeneration

It has been suggested that neuronal pathology could be affected by neuron-autonomous GALC, independently from demyelination or neuroinflammation. Along this line, GALC is highly expressed in neurons (Dolcetta et al., 2004; Weinstock, Kreher, et al., 2020;

Zhang et al., 2014) and neuronal pathology in GLD occurs before distinct demyelination in the *twitcher* and *twi-5j* mouse models (Cappello et al., 2016; Castelvetti et al., 2011; Potter et al., 2013). Neuronal swellings and varicosities were specifically documented in *twitcher* spinal cords at postnatal day 7, prior to overt CNS myelination (Castelvetti et al., 2011). Similarly, purified primary neurons from *twitcher* have abnormal neurites and axonal transport, attributed to some degree of autonomous psychosine production (Castelvetti et al., 2011). GALC has also been proposed to have a role in the maintenance of subventricular zone neurogenesis during the early postnatal period (Santambrogio et al., 2012). Interestingly, it was found that neuronal pathology was still present when *twitcher* mice were crossed to knockout mice for ceramide galactosyltransferase (Ezoe et al., 2000a), the enzyme that synthesizes galactosylceramide (and therefore upstream of psychosine formation). This suggests that neuronal GALC may function on a substrate and in a role unrelated to that of myelin and GalCer turnover. Nonetheless defining the autonomous role of GALC in neurons has been technically challenging using conventional global knockout or mutant models.

The new *Galc* conditional knockout mouse identified a key developmental period that requires GALC prior to myelination; exposing a critical period of vulnerability to GALC ablation between postnatal day 4–6 in mice (Weinstock, Kreher, et al., 2020). Early *Galc* deletion before postnatal day 4, results in a worse Krabbe phenotype, higher psychosine in the hindbrain, and a shorter life-span of the mice. Neurons in the hindbrain have more axonal degeneration when GALC is deleted early, in line with defective neurogenesis of the hindbrain found in a zebrafish model of GLD (Zizioli et al., 2014). During this vulnerable period, GALC is expressed in all brain cells, with the highest expression in neurons. Furthermore, neuronal GALC is involved in the development and maturation of immature T-box-brain-1 (TBR1) positive brainstem neurons, suggesting that brainstem development is affected by intrinsic neuronal GALC (Weinstock, Kreher, et al., 2020). This developmental role of GALC may correlate with the observed benefit of early therapeutic interventions for *twitcher* and Krabbe patients. Therefore, augmenting GALC levels before this defined period could possibly increase the efficacy of GLD therapy.

The mechanisms underlying the unique biological role of GALC in neurons remains unknown. Lysosomes are essential for formation of synaptic structures including dendritic spines (Vukoja et al., 2018). In neurons, lysosomes are present in dendrites and traffic bidirectionally, regulating dendritic spine number in an activity-dependent manner (Goo et al., 2017). Lysosomes control the excitatory synaptic signal strength by modulating the fate of the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and sorting them for degradation or recycling (Fernández-Monreal, Brown, Royo, & Esteban, 2012). In hippocampal neurons, activity-dependent release of lysosomal Cathepsin B regulates the structural plasticity of dendritic spines by triggering MMP-9 activation and extracellular matrix remodeling (Padamsey et al., 2017). Therefore, it is possible that the aberrant synapse, caused by GALC-deficient lysosomes, may impact neuronal activity and function, eventually causing neuronal death, and abnormality in brain development (Hensch, 2005; Marín, 2016). In addition, if brainstem neurons do not fully mature, they could be vulnerable to accumulated psychosine, less responsive to dynamic signals, and eventually eliminated (Pfisterer & Khodosevich, 2017; Weinstock, Kreher, et al., 2020).

Further investigation is required to understand the specific cellular events which require GALC for neural development and if similar mechanisms are present in other LSDs.

An additional theory relevant for neuronal pathogenesis in GLD is in the metabolism of a minor and lesser-studied substrate of GALC, lactosylceramide (Wenger, Sattler, & Hiatt, 1974). Lactosylceramide is synthesized by β -1,4-galactosyltransferase 6 in astrocytes during chronic CNS inflammation, but has been documented to contribute to neurodegeneration (Mayo et al., 2014), perhaps related to its regulation function of TNF- α induced astrogliosis (Pannu, Singh, & Singh, 2005). In the experimental autoimmune encephalomyelitis (EAE) model, sphingolipid lactosylceramide stimulates astrocytes (Mayo et al., 2014) and recruits microglia and infiltrating macrophages. In the canine GLD model, lactosylceramide was highly elevated in the cerebrospinal fluid and further increased with age, in addition to psychosine and GalCer (Corado et al., 2020), indicating a possibility of the contribution of accumulated lactosylceramide to GLD pathology.

4 | SECONDARY PERTURBATIONS FROM GALC DYSFUNCTION

4.1 | Lysosome accumulation and dysfunction

In most LSDs, a number of common pathologic molecular cascades occur at the cellular level as substrates accumulate leading to impaired lysosomal function. Lysosomes have multiple roles in cellular homeostasis including the regulation of endocytosis and autophagy via nuclear signal transduction as well as plasma membrane repair and exocytosis. All of these aspects of lysosomal biology are not surprisingly susceptible to impairments in LSDs. For example, under physiologic conditions there must be a sufficient number and volume of lysosomes to meet the metabolic requirements of the cell. The number of lysosomes varies from 50 to 1,000 per cell (H. Xu & Ren, 2015), and occupies 1–15% of cell volume (Marzella, Ahlberg, & Glaumann, 1982). These factors change dynamically in accordance with cellular demands (such as starvation) both transcriptionally and post-transcriptionally (Eriksson, Wäster, & Öllinger, 2020; Settembre et al., 2011). Instead, the cellular space occupied by lysosomes in LSDs increases drastically. Similarly, the pH of the lysosomal lumen is normally carefully maintained via the vacuolar ATPase (Platt, Boland, & van der Spoel, 2012). Substrate accumulation in LSDs can influence the regulation of lysosomal pH (Folts et al., 2016; Holopainen, Saarikoski, Kinnunen, & Järvelä, 2001). For example, lysosomes in the oligodendrocytes of GALC-deficient *twitcher* are enlarged and have higher pH. Psychosine has also been shown to disrupt lysosomal function by antagonizing the lysosomal vacuolar-type H⁺-ATPase (Tapper & Sundler, 1995), suggesting a change in lysosomal pH due to psychosine accumulation. Therefore, it has been suggested that re-acidification of lysosomal pH may ameliorate Krabbe pathogenesis (Folts et al., 2016).

There is also a growing body of evidence that suggests that distended lysosomes in LSDs, which often contain copious substrate, are structurally sensitive and prone to membrane rupture (reviewed in Wang, Gómez-Sintes, & Boya, 2018). This process is often referred to as LMP, and can potentially lead to cell death. If the lysosomes rupture, acidic hydrolases, including cathepsins, are released to the cytoplasm and have the potential to degrade cellular components. Their hydrolysis activity seems to continue to work to some degree in cytoplasmic pH, resulting in apoptosis or necrosis depending on the degree of

permeabilization (Boya & Kroemer, 2008). LMP seems to be a common pathophysiologic mechanism in LSDs, though its specific role in GLD has yet to be explored.

In addition to perturbed lysosomal function, the LSDs can also cause dysfunction in the broader endosome-lysosome network. Substrate delivery to the lysosome occurs via well-coordinated steps of cargo formation and delivery. These include cargo recognition, autophagosome and endosome formation, fusion with the lysosome and subsequent recycling of proteins and lipids. Psychosine exposure to OLs caused abnormalities in endo-lysosomal transport, as proven by a decreased endocytic import. Furthermore, once the larger endosome trafficking system is perturbed, secondary accumulation of additional, biochemically unrelated, metabolites can also occur (Walkley & Vanier, 2009). In turn, this compounding effect can affect the integrity of other sub-organelles.

In addition, lysosome-mediated exocytosis regulates the proper targeting of proteins residing in vesicles, the plasma membrane, or extracellular space. Therefore, dysfunctional lysosomes can mis-target those proteins to the wrong place or the ER which could activate the unfolded protein response (UPR) and oxidative stress, which have been suggested as a key mechanism of Krabbe pathogenesis (Irahara-Miyana et al., 2018). Furthermore, toxic effect of misfolded GALC protein itself due to mutations and associated polymorphisms can generate similar ER or oxidative stresses (Irahara-Miyana et al., 2018; Shin et al., 2016), which may trigger neuroinflammation by attracting and activating immune cells (Formichi et al., 2007; Santambrogio et al., 2012). Finally, it is conceivable that dysregulated autophagy is involved in the inflammatory response. Indeed, autophagy modulates inflammation for normal host-pathogen defense, by controlling adaptive immunity through regulation of antigen presentation.

4.2 | Autophagic dysfunction

Autophagy is a specialized “self-eating” cellular function, which eliminates unnecessary or damaged cellular components, providing nutrient and metabolic homeostasis. (Ravikumar et al., 2010). There are three types of autophagic pathways: macroautophagy, chaperone-mediated (CMA), and microautophagy. All three types use different routes to reach lysosomes for the degradation of autophagy-targeted materials. Macroautophagy (commonly referred to simply as “autophagy”) sequesters materials into double-membrane-layer bound autophagosomes and then fuses with lysosomes to form autophagolysosomes. The lipidated microtubule-associated protein light chain 3 (LC3-II) is involved in all parts of the autophagy process. p62 is another indicator of autophagic flux, because it interacts with autophagic substrates and delivers them to autophagosomes for degradation (Rusten & Stenmark, 2010). Autophagosomes are dispersed along microtubules in the cytosol to reach to and fuse with lysosomes, forming autolysosomes (Kast & Dominguez, 2017) where all of the cargo in autophagy is degraded and/or recycled for energy and membrane components. CMA is a selective autophagic pathway that only targets proteins having a KEFERQ motif or a similar sequence for degradation (Kaushik et al., 2011). Heat shock protein 70 recognizes and binds to the proteins destined for CMA, and docks on lysosomes via interaction with LAMP-2A, allowing lysosomal engulfment (Venugopal et al., 2009).

Microautophagy is mediated by direct pinocytosis of cytosolic materials into lysosomes (Sahu et al., 2011).

Due to the intimate relationship of autophagy with lysosomal pathways, defects in autophagy are often secondary to the abnormally high accumulation of various lysosomal metabolites in most LSDs (Lieberman et al., 2012). In LSDs, autophagosome-lysosome fusion is impaired, which results in limited capacity for autophagic flux (Kollmann et al., 2012). Ultimately, the autophagosome is slowly processed by lysosomes, which results in the accumulation of autophagic substrates, LC3-II positive vacuoles, as well as ubiquitinated and aggregate-prone molecules in the cytoplasm, including p62 and alpha-synuclein (Ravikumar, Duden, & Rubinsztein, 2002; Teil et al., 2020). Since autophagy also has a role in the maintenance of mitochondria quality by selectively degrading dysfunctional mitochondria via mitophagy (Kim, Rodriguez-Enriquez, & Lemasters, 2007), impaired autophagic flux can lead to the increase of defective mitochondria. Similarly, lysosomes themselves have a quality control mechanism that relies on autophagy, termed lysophagy (Papadopoulos, Kravic, & Meyer, 2020). Therefore, lysosomal dysfunction, which can impair autophagic machinery, may further limit the normal cellular ability to repair damaged lysosomes via lysophagy.

Increased levels of LC3-positive autophagosomes have been observed in GALC-deficient oligodendrocytes (Ribbens et al., 2014). Abnormally large p62 cytoplasmic aggregates are also found in the brain and sciatic nerve of *twitcher* (Del Grosso et al., 2019). Co-accumulation of p62/LC3-II aggregates are mostly present in white matter regions undergoing demyelination and neuroinflammation (D. S. Lin et al., 2020). It is thought that psychosine impairs autophagic flux by affecting the formation steps of autophagosomes and autophagolysosomes (Del Grosso et al., 2016). Not surprisingly, there is also data from GLD tissues that show dysfunction in a complimentary protein degradative system known as the ubiquitin-proteasome system (D. S. Lin et al., 2020). Similarly, GALC deficient macrophages exposed to GalCer expressed markers of the closely related integrated stress response including CHOP, ATF4, and GADD34 (Weinstock, Shin, et al., 2020). Overall, it appears that GALC deficiency causes a broad cellular consequence in pathways involving metabolic regulation including autophagy and other protein quality control mechanisms. Suboptimal functioning of these important pathways may even have theoretical consequences in GALC haplo-insufficiency. For example, a strong association has now been developed between GBA1 haplo-insufficiency and the risk for neurodegenerative disorders like Parkinson's disease (reviewed in Migdalska-Richards & Schapira, 2016). The link to neurodegenerative diseases for GALC, and other lysosomal hydrolases, is therefore of growing significance (Robak et al., 2017).

4.3 | Progressive inflammation

High levels of inflammation in both the CNS and PNS is a prominent feature of GLD, documented by widespread microgliosis, CNS macrophage infiltration and expression of proinflammatory chemokines (LeVine, Wetzel, & Eilert, 1994). While microgliosis and globoid cell infiltration is clearly an important aspect of GLD pathology, there remains some question regarding the degree to which this process drives pathogenesis. There

exists a longstanding correlation between globoid cell accumulation and areas of overt demyelination (Taniike & Suzuki, 1994), which was conventionally interpreted to signify that globoid cells form in areas of demyelination and myelin debris. However, some studies suggest that neuroinflammation precedes overt myelin loss (Potter et al., 2013; Santambrogio et al., 2012; Snook et al., 2014). For example, the *twi-5j* mouse model harboring Galc-E130K mutation had robust neuroinflammation in the absence of demyelination, showing an active gliosis reaction as early as 2 weeks postnatally, before demyelination. In addition, proinflammatory cytokines such as CCL2, CCL3, CCL5, CXCL1, CXCL10, IL1b, IL6, TNF-alpha, and toll-like receptor 2 (TLR2) are upregulated in presymptomatic *twitcher* brains starting postnatal 2 weeks old and increased exponentially with disease progression (Santambrogio et al., 2012; Snook et al., 2014). Similarly, astrogliosis also precedes overt demyelination and coincides with macrophage infiltration, arguing that astrocytes have a compounding role in the disease progression of GLD (Ijichi et al., 2013), possibly via over-expression of prostaglandin D signaling (Mohri et al., 2006).

Due to the assumption that proinflammatory conditions drive GLD pathogenesis, a number of attempts have used anti-inflammatory therapeutic approaches in *twitcher* mice. For example, the anti-inflammatory agent ibudilast was shown to modestly improve GLD by delaying symptom progression and demyelination (Kagitani-Shimono et al., 2005). Similarly, the S1P agonist fingolimod (FTY720), which acts by preventing lymphocyte egression from lymph nodes, was found to attenuate psychosine-induced glial cell death and demyelination both in vitro and ex-vivo models of *twitcher* mice (Béchet et al., 2020; O'Sullivan & Dev, 2015). Nonetheless the overall efficacy of anti-inflammatory agents alone on preventing GLD pathogenesis has been disappointing. Similarly, genetic crosses of *twitcher* mice with TNF-*alpha* R1 knockout mice did not significantly alter disease progression (Pedchenko, Bronshteyn, & LeVine, 2000) and crossing *twitcher* mice with MHC-II knockout mice had no effect on survival (Matsushima et al., 1994). These data further contributed to the question regarding the significance of neuroinflammation in GLD.

Furthermore, despite the evidence for a proinflammatory state being a driving force for GLD pathogenesis, there exists some evidence that macrophages may even be protective in GLD. When the *twitcher* mouse was crossed to a macrophage-deficient osteopetrotic background, which harbor mutations in *Csf-1*, the mice had a more severe phenotype, suggesting that macrophages play a protective role (Y. Kondo, Adams, Vanier, & Duncan, 2011). Our recent study showed that macrophages that express GALC are, in fact, neuroprotective and limit the overt demyelination, axonal degeneration, and clinical decline seen in global *Galc* knockout tissues (Weinstock, Shin, et al., 2020). We suspect that this effect is mediated by neuroinflammation elicited from myelin substrates in *Galc* deficient macrophages (i.e., globoid cells). This data is in line with a study in which *Galc* deficient oligodendrocytes were transplanted into the demyelinating *shiverer* mice, which are normally unable to myelinate effectively. *Shiverer* mice with transplanted *Galc* deficient oligodendrocytes were able to differentiate and myelinate normally (Y. Kondo et al., 2005), suggesting that the demyelinating phenotype of GLD is strongly influenced by non-oligodendrocyte conditions. Thus, the contrasting observations regarding a protective versus deleterious effects of innate immunity in GLD can be reconciled by the view that GALC positive macrophages are beneficial for GLD, while GALC deficient macrophages aggravate the disease.

5 | THERAPIES FOR GLD

Due to the authentic nature of the *twitcher* model of GLD, a stunning number of therapeutic strategies have been tested on *twitcher* mice (reviewed in Mikulka & Sands, 2016). These include bone marrow transplantation (BMT) or HSCT (Ichioka, Kishimoto, Brennan, Santos, & Yeager, 1987; Yeager, Brennan, Tiffany, Moser, & Santos, 1984), neural and mesenchymal stem cell transplantation (Neri et al., 2011; Ripoll et al., 2011; Strazza et al., 2009; Taylor & Snyder, 1997), substrate reduction therapy (LeVine, Pedchenko, Bronshteyn, & Pinson, 2000), antioxidant therapy (Pannuzzo et al., 2010), pharmacological chaperone therapy (Berardi et al., 2014), enzyme replacement therapy (ERT; Lee et al., 2005), and gene therapy (GT) (Lattanzi et al., 2010; Lee et al., 2007; D. Lin et al., 2005; Meng, Shen, Watabe, Ohashi, & Eto, 2005; Rafi et al., 2012, 2015; Rafi, Luzi, & Wenger, 2021; Shen, Watabe, Ohashi, & Eto, 2001). Additionally, a large number of combination therapies have been tested (Galbiati et al., 2009; Karumuthil-Meilethil et al., 2016; D. Lin et al., 2007; Rafi et al., 2015; Rafi, Luzi, & Wenger, 2020; Reddy et al., 2011; Ricca et al., 2015).

6 | ENZYME REPLACEMENT THERAPY

The basis for enzyme replacement therapy (ERT) for lysosomal diseases is cross-correction, the phenomenon by which lysosomal enzymes (including GALC), are secreted from enzyme-producing cells and taken up by enzyme-deficient cells (Sands & Davidson, 2006), or supplied exogenously by therapeutic intervention. ERT has been successfully used in a number of LSDs including Gaucher's disease (Schiffmann et al., 1997), Fabry's disease (Germain, 2002), MPS-I (Pasqualim et al., 2015), and others, though peripheral disease seems easier to treat than neurological defects. This phenomenon is thought to highlight the difficult of delivering large proteins across the blood brain barrier (Thomas & Kermode, 2019). The molecular basis for ERT is that secreted lysosomal hydrolases are tagged with mannose-6-phosphate residues and taken up by M6P receptors (Coutinho et al., 2012). Human GALC has four potential *N*-glycosylation sites (Deane et al., 2011), though which site is required for activity is currently unknown.

Cross-correction of GALC was shown to occur, *in vitro*, in GLD fibroblasts from conditioned media and was inhibited by M6P (Nagano et al., 1998). This same process also occurs, *in vitro*, when using conditioned-media from fibroblasts transduced with lentiviral GALC cDNA (Rafi, Fugaro, et al., 1996). GALC-deficient oligodendrocytes were also able to take up exogenous GALC enzyme (Luddi et al., 2001), *in vitro*. An argument for evidence of *in vivo* cross correction of GALC to oligodendrocytes comes from a study that showed that *twitcher* oligodendrocytes transplanted into *shiverer* mice (myelin-deficient) were able to myelinate properly (Y. Kondo et al., 2005). While the authors concluded that *twitcher* oligodendrocytes received GALC from their environment, it is also possible that GLD pathogenesis relies on the role of GALC in cells other than oligodendrocytes, like microglia (discussed in Nicaise et al., 2016), macrophages, neurons and astrocytes, as suggested by recent data using cell-specific *Galc* (Weinstock, Shin, et al., 2020).

In general, GALC ERT has been disappointing in GLD and only seemed to improve survival in *twitcher* mice by a few days (Lee et al., 2005). Some speculated that this problem may

have been: (a) due to poor ability of the recombinant enzyme to cross the blood–brain barrier (BBB) or (b) due to an immune response produced against the recombinant enzyme (Won, Singh, & Singh, 2016). The immune response was particularly pertinent because the *twitcher* mouse is a full null mouse and therefore may recognize injected recombinant GALC as foreign. To overcome this issue, one study generated a humanized mouse model of GLD using a knock-in of a human GLD mutation in the *Galc* locus (Matthes et al., 2015). While intracerebral injection of recombinant human GALC did not trigger an immune reaction and bypassed the BBB, there remained no improvement to the phenotype of the GLD mice. The conclusion, was that cross-correction of in vivo GALC to oligodendrocytes was not efficient (Matthes et al., 2015). In line with this data, a GT study showed that immunostaining for recombinant GALC was always restricted to transduced cells and was not able to cross-correct neighboring cells (Meng et al., 2005). Most recently, our study showed that in vitro GALC deficient cells, including both primary mouse Schwann cells and human patient fibroblasts, were less capable of receiving M6P-tagged GALC than their wildtype counterparts (Weinstock, Shin, et al., 2020). We also showed poor in vivo cross correction of GALC in Schwann cells.

Despite these limitations, a number of promising approaches have been directed toward improving GALC delivery and uptake by *twitcher* neural tissues. For example, GALC has recently been encapsulated into poly-(lactide-co-glycolide) nanoparticles, functionalized with brain targeting peptides (Del Grosso et al., 2019). While this study showed promising in vivo GALC delivery to brains of *twitcher* mice, functional and clinical studies were not explored. A second group recently developed chimeric GALC enzymes engineered to express unique peptide domains hypothesized to improve secretion, uptake, and transport across the BBB (Ricca, Cascino, Morena, Martino, & Gritti, 2020). In particular, GALC fusion proteins to the signal peptide of iduronate-2-sulfatase (IDS) or the low-density lipoprotein receptor (LDLr)-binding domain (from ApoE II) were generated and produced by LV-transduced cells. Cells producing IDS-GALC had improved secretion of enzyme, compared to GALC-only producing cells, and similar or improved cross-correction of in vitro *twitcher* cells. Furthermore, the ApoE-GALC approach has the additional opportunity to leverage increase LDLr protein expression (and therefore increased cross-correction capabilities) via statin pharmacotherapy (Ricca et al., 2020). In summary, while recent works have shed light on the limitations of GALC cross-correction and the delivery of ERT across the BBB, exciting novel approaches continue to be developed to facilitate enzyme replacement in GLD and other LSDs.

7 | HEMATOPOIETIC STEM CELL THERAPY

The first example of HSCT for LSDs was published in the Lancet in 1981 and showed reversal of clinical features of Hunter's disease following transplantation (Hobbs et al., 1981). In the wake of this marquis paper, a number of groups were trying to test this paradigm in vivo, which presented challenges as some LSD mouse models were not severe enough to test survival (Hong, Sutherland, Matas, & Najarian, 1979), while others that were severe showed no improvement in survival (Miyawaki, Mitsuoka, Sakiyama, & Kitagawa, 1982). In 1984, a landmark study by Yeager and Santos was published that showed that HSCT delivered to irradiated *twitcher* mice could remarkably extend average survival from

40 to 80 days (Yeager et al., 1984). A follow up study showed that GALC activity was normalized in HSCT treated mice (Ichioka et al., 1987), and that psychosine production was drastically delayed in the CNS of mice ultimately reaching 30–35% of untreated levels at the time of death (Ichioka et al., 1987). PNS psychosine was also initially reduced, although it did accumulate to untreated levels by the time of death (Ichioka et al., 1987).

A repeat study, using donor mice genetically identified with a unique MHC isoform, confirmed the pronounced survival and psychosine reduction in HSC-treated *twitcher* mice (Hoogerbrugge et al., 1988). Furthermore, some donor derived bone marrow cells were found in *twitcher* brains, which could explain the increased GALC activity in the brain. Strikingly, the number of globoid cells continued to decrease in brains of grafted mice, while the number of “foamy macrophages” continued to increase. These foamy macrophages lacked the typical GalCer inclusions of globoid cells and were thought to reflect GALC-expressing donor macrophages involved in myelin turnover (Hoogerbrugge et al., 1988). A more in-depth analysis of these treated mice (A. Kondo et al., 1988; Suzuki & Suzuki, 1990) showed that there were areas of remyelination found throughout the CNS and PNS, which were attributed to the presence of GALC-expressing donor macrophages. While the mechanisms of HSCT was originally considered to occur via cross-correction, recent studies suggest that the beneficial properties may instead be from restored phagocytic function of donor macrophages, which retain GALC function (Weinstock, Shin, et al., 2020; Wolf et al., 2020).

Interestingly, the disease process in BMT-treated *twitcher* mice continued to progress in the CNS and PNS despite measurable improvement in the enzymatic activity after BMT. In fact, at the ultrastructural level, GalCer inclusions were still evident in oligodendrocytes and Schwann cells throughout survival of mice. Overall, it was suggestive that “the metabolic defect in the *twitcher* mouse still remained long after BMT” (A. Kondo et al., 1988). This data further argues that the therapeutic benefit of HSCT is likely through restoring macrophage function rather than supplying GALC to myelinating glia. Additional studies showed that BMT was effective not only in reducing globoid cells, but also in decreasing widespread markers of inflammation including TNF α (Wu et al., 2001). Along these lines, a more recent study showed that BMT was even more effective at inducing anti-inflammatory effects than the direct action of anti-inflammatory drugs (Luzi et al., 2009).

Initial human clinical trials for HSCT in GLD were first published in 1998 (four Late-Onset GLD, LOGLD, and one presymptomatic Early Infantile GLD, EIGLD; Krivit et al., 1998). Preliminary findings found that HSCT was efficacious as the four LOGLD patients had a reversal in CNS deterioration, as measured by decreased CSF protein and improved myelination by MRI, while the EIGLD patient had delayed symptom development. A 2005 study then showed that HSCT was particularly helpful if delivered to presymptomatic EIGLD patients but was not efficacious in postsymptomatic EIGLD patients (Escobar et al., 2005). This landmark study emphasized the urgency of secure presymptomatic diagnosis for EIGLD and launched a host of clinical research including the implementation of newborn screening among several states as well novel studies to help better diagnose GLD. A follow up study of 18 EIGLD patients that received HSCT found that though most patients do significantly better than the natural history of EIGLD, all patients are abnormal and continue

to develop disease progression both in the CNS and PNS (Wright, Poe, DeRenzo, Haldal, & Escolar, 2017).

8 | GENE THERAPY

The *twitcher* model is one of oldest mouse models used for GT and has been tested with a number of viral delivery systems. Viral GT contains two components: the vehicle, which is comprised of a viral vector that is emptied of viral genes, and the genetic cargo, which in this case is *GALC* cDNA (often species matched to reflect the host species). The viral vehicles used for GT on *twitcher* mice have been broad and include adenovirus, lentivirus, and adeno-associated virus (AAV; Lee et al., 2007; D. Lin et al., 2005; Meng et al., 2005; Rafi et al., 2012, 2015; Shen et al., 2001). More recent approaches have used AAVrh10 (Rafi et al., 2012) or AAV9 (Marshall et al., 2018; Pan et al., 2019) serotypes, thought to have ideal transduction in brain and increased tropism for glia, without genome integration and minimal risk of inducing malignancy. It should be noted that lentiviruses, which integrate within the genome, are also of use particularly in the case of ex vivo transduction of hematopoietic stem cells, which have been very successful in the clinic (Aiuti et al., 2013; Biffi et al., 2013; Sessa et al., 2016).

Although viral GT should theoretically be curative, the efficacy in vivo has had major limitations. *twitcher* studies using viral-delivered GT tend to only modestly improve survival, typically by approximately 1.5–2-fold. Therefore, many approaches have been made to improve delivery and transducing ability. For example, some studies use tail-vein injections (Rafi et al., 2012), while others use intrathecal or intracerebral injections (D. Lin et al., 2005) while still others use multiple sites of injection (Rafi et al., 2012). Dose of virus is also very important, as a recent article had far better success in the *twitcher* mouse by using significantly higher doses of AAV9 (Marshall et al., 2018). It should also be noted, that AAV vectors have particularly strong tropism for neurons (Asokan, Schaffer, & Samulski, 2012) compared to glia, and so neurons need to provide a significant supply of GALC for other cell types via cross-correction (Mikulka & Sands, 2016). Therefore, discussions on improving cross-correction of GALC are relevant not just for ERT or HSCT, but also for viral GT. In addition, the oncogenic potential of AAV-vector should be clearly addressed before considering human trials (Li et al., 2021).

9 | SUBSTRATE REDUCTION THERAPY

The idea of a pharmacological inhibitor of psychosine synthesis is of major interest in GLD. For example, the medication L-cycloserine has been shown to moderately reduce psychosine accumulation and GLD-associated toxicity in *twitcher* mice (LeVine et al., 2000). This medication, though toxic in its own right, has been found to be beneficial when used in conjunction with other forms of therapy (Ricca et al., 2015). Current groups are working to develop high-throughput screens to develop novel agents to inhibit psychosine production (Ribbens et al., 2014). Of note, genetic models of CGT/GALC double knockout mice found that reducing GalCer/Psychosine production was beneficial to some degree (Ezoe et al., 2000b), though total ablation of CGT was not curative (Ezoe et al., 2000a). Novel enzymatic targets for substrate reduction inhibitors now include the enzyme acid ceramidase, which

could prevent the formation of psychosine via the degradative route (Li et al., 2019). For example, the chemotherapeutic agent carmofur, which directly inhibits acid ceramidase activity, reduced psychosine production and modestly extended life span of *twitcher* mice that were haplo-insufficient for *Asah1* (Li et al., 2019).

10 | COMBINATION THERAPY

Due to the partial effect of many different modalities of therapies, a number of groups have approached the concept of combining different modalities of therapy (reviewed in Ricca & Gritti, 2016). The effect of multimodal therapy has had profound improvements of survival on both the *twitcher* mouse model of GLD, as well as on the dog model of GLD. In particular, the use of HSCT has been particularly efficacious in synergizing with viral-directed GT. The synergistic benefit of combining HSCT and GT has been replicated by many different labs with different viral vectors and regimens of delivery (Hawkins-Salsbury et al., 2015; D. Lin et al., 2007; Qin et al., 2012; Rafi et al., 2020; Reddy et al., 2011).

Work in uncovering the mechanism behind this synergy revealed that HSCT seems to have a profound immunomodulatory benefit that is not replicated by GT (Reddy et al., 2011). This effect seemed to be independent from the cross-corrective ability of circulating stem cells. This concept was highlighted by the fact that other stem cell therapies, like neural stem cell therapy (NSCT) or embryonic stem cell derived OLs, which should be able to cross-correct, were not efficacious in *twitcher* mice (Kuai et al., 2015; Ricca et al., 2015). Instead, when NSCT was given with HSCT, there was once again robust synergy similar to GT + HSCT, emphasizing the unique benefit of HSCT (Ricca et al., 2015). Taken together, these data are in agreement with the idea that HSCT may work independently of its ability to increase the amount of GALC activity in tissues. Similarly, the efficacy of ICV Mesenchymal stem cells in *twitcher* mice seemed to have an immunomodulatory effect, though not all cytokine levels were returned to normal (Ripoll et al., 2011).

Additional forms of “combination therapy” include the concept of augmenting HSCT precursor cells ex-vivo prior to transplantation. This strategy is particularly appealing for metabolic disease as patient-treated HSCs can be genetically corrected by (CRISPR-Cas editing) or transduced to overexpress the missing enzyme by lentiviral GT. The rationale for this approach is two-fold: (a) by inducing the patient’s own HSCs to produce the missing lysosomal enzyme the severe risk of allogeneic transplantation would be avoided and (b) it would theoretically be possible to produce HSCT-derived cells that produce supra-physiologic levels of GALC that are even more efficient at correcting metabolic defects. This approach has remarkable effects in ARSA deficiency of metachromatic leukodystrophy patients (Biffi et al., 2013; Sessa et al., 2016). In light of recent data that shows limitations of cross-correction from HSCT in MLD patients (Wolf et al., 2020), it would be intriguing to evaluate if overexpressed GT-HSCT has improved cross-correctable capabilities. This approach would, in theory, be particularly appealing for GLD, where functional impairment of GALC deficient hematopoietic stem cells (Visigalli et al., 2010) has been described. Unfortunately, GALC overexpression in HSCs seems to be toxic, perhaps related to changes in the bioactive sphingolipids ceramide, sphingosine and sphingosine-1-phosphate (Costantino-Ceccarini et al., 1999). Novel alternative approaches have therefore considered

modulating GLD HSCs using for miRNA GT that would increase GALC expression (Gentner et al., 2010).

11 | CLOSING REMARKS

For over 50 years, physicians and scientists have made significant advances in understanding the molecular mechanisms of GLD, ranging from proposing the psychosine hypothesis (reviewed in Suzuki, 1998) to cloning *GALC* (Chen, Rafi, de Gala, & Wenger, 1993). Translational advances have also emerged as HSCT is the standard of care for presymptomatic patients with GLD (Escobar et al., 2005) and may be positively augmented by co-administration with viral GT (D. Lin et al., 2007). Nonetheless, the past year has marked a number of significant discoveries that seem to shed light on key unsolved questions in GLD, including the biochemical formation of psychosine from acid ceramidase (Li et al., 2019), the cellular origin of psychosine from myelinating glia (Mikulka et al., 2020; Weinstock, Shin, et al., 2020), the developmental role of GALC in neurons (Weinstock, Kreher, et al., 2020), and the beneficial effect of GALC expressing macrophages on remyelination and neuroprotection (Weinstock, Shin, et al., 2020). Many of these discoveries have been advanced with the development of novel tools, including genetic mouse models.

Despite these exciting discoveries, a number of important questions remain: Can acid ceramidase inhibitors be modulated to reduce toxicity, while remaining beneficial in patients with GLD? Are mechanisms of oligodendrocytes/microglia analogous to Schwann cells and macrophages? Do astrocytes (or neurons) of the CNS play an important part? Can cross correction be improved in GALC-deficient cells? Does the amount of enzyme available influence cross correction? Why does GALC secretion and transfer occur in physiology? Are the defects of cross correction reflective of other LSDs? If psychosine is generated by ASAH1 in lysosome, are the concentrations higher there? If so, does toxicity start at lysosome? Do the crystals evident by electron microscopy in GLD tissues contribute to pathology? What exactly are they composed of and how are they formed? For some time, researchers have been trying to optimize cell and tissue culture strategies to best represent normal and disease physiology, and one such research group has recently proposed the use of human induced pluripotent stem cells (hiPSCs) in developing 2D and 3D co-cultures to foster neuron-glia functional and dysfunctional interactions in order to study and define cell-specific role in neurodevelopment, neuroinflammation, and progression of the disease (Luciani, Gritti, & Meneghini, 2020). Such approaches might prove beneficial and fitting to answer some of the questions mentioned above.

The advances in GLD also have an impact on broader diseases. A recent discovery linked the association of GBA1 carrier mutants, responsible for the LSD Gaucher's disease, with increased risk for Parkinson's disease. It appears that mutations in GALC and other lysosomal enzymes have similar risk associations for neurodegenerative disease (Chang et al., 2017) and multiple sclerosis (Sawcer et al., 2011). These fascinating observations have, in turn, led to further unanswered questions in the field: how does GALC haploinsufficiency contribute to more common disorders of adulthood? Is there a unifying mechanism connecting lysosomal hydrolase deficiency and neurodegeneration? Can LSD treatments be

beneficial in LSD carriers who develop neurodegenerative disease? While answers to these questions are likely to lead to new hypotheses, they emphasize the exciting nature of recent advances in the field and the broader application to common neurologic disorders.

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DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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FIGURE 1.

Galactosphingolipid biology in myelinating oligodendrocyte. The myelin sheath is a compact layer of lipid-rich membranes produced by oligodendrocytes in the central nervous system and by Schwann cells in the peripheral nervous system. Galactosylceramide (GalCer), a glycosphingolipid synthesized by ceramide synthase (CERS2) and ceramide galactosyl transferase (CGT), is a major component of myelin, present in the extracellular side of the membrane (so-called intraperiod line), see inset. GalCer can be further processed into sulfatides by addition of a sulfate group (red dots) by cerebroside sulfotransferase (CST). GalCer is catabolized into galactose (blue hexagons) and ceramide by galactosylceramidase and saposin A in the lysosome. Deficiency of these enzymes cause globoid cell leukodystrophy. CERS2, Ceramide synthase 2; CGT, ceramide galactosyl transferase; CST, cerebroside sulfotransferase; MBP, myelin basic protein; PLP, proteolipid protein

FIGURE 2.

Cell-specific pathophysiology in the CNS of globoid cell leukodystrophy. “GALC +” area depicts cells that express galactosylceramidase enzyme and are physiologically normal. “GALC Null” area contains cells that lack galactosylceramidase enzyme and represent globoid cell leukodystrophy (GLD). GALC Null oligodendrocytes produce psychosine from GalCer via acid ceramidase, undergo diffuse loss of myelin, and contribute to secondary axonal degeneration. Psychosine in neurons causes axonal degeneration. Microglia and globoid cells are unable to digest myelin (GalCer) efficiently, accumulate cytosolic crystals and become proinflammatory, further contributing to axonal degeneration and compromising remyelination. Astrocytes become activated, presumably in response to the surrounding pathology

FIGURE 3.

Demyelination, axonal degeneration and globoid cells in the nervous system of GLD-Krabbe patients. (a) Luxol-Fast-Blue, periodic acid schiff (PAS) staining of the cerebellar white matter from a GLD patient shows profound demyelination (absence of blue-stained myelin), neurodegeneration (low density of purple stained axons, arrows) and Globoid cells filled with PAS positive glycolipids storage (arrowhead). (b) Epon semithin sections of brachial plexus nerve from a globoid Leukodystrophy patient show very low density of myelinated fibers, myelinated fibers with onion bulbs (arrowhead) and many demyelinated fibers (double arrowheads), all indicative of chronic demyelination. Fiber density is also low, suggestive of chronic axonal degeneration. A globoid cell macrophage is indicated with an asterisk. Tissue (a) and image (b) courtesy of Dr. Julia Kofler and the NDRD brain and tissue bank at the University of Pittsburgh and Krabbe Partners for Research