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Role of Endogenous Psychosine Accumulation in Oligodendrocytes Differentiation and Survival: Implication for Krabbe Disease

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Abstract

Krabbe disease is a lethal, demyelinating condition caused by genetic deficiency of galactocerebrosidase (*GALC*) and resultant accumulation of its cytotoxic substrate, psychosine (galactosylsphingosine), primarily in oligodendrocytes (OLs). Psychosine is generated by galactosylation of sphingosine by *UDP-galactose:ceramide galactosyltransferase* (*CGT*), a galactosylceramide synthesizing enzyme which is primarily expressed in OLs. The expression of *CGT* and the synthesis of galactosyl-sphingolipids are associated with the terminal differentiation of OL, but little is known about the participation of endogenous psychosine accumulation in OL differentiation under *GALC* deficient conditions. In this study, we report that accumulation of endogenous psychosine under *GALC* deficient Krabbe conditions impedes OL differentiation process both by decreasing the expression of myelin lipids and protein and by inducing the cell death of maturing OLs. The psychosine pathology under *GALC* deficient conditions involves participation of secretory phospholipase A₂ (sPLA₂) activation and increase in its metabolites, as evidenced by attenuation of psychosine-induced pathology by treatment with pharmacological inhibitor of sPLA₂ 7,7-dimethyleicosadienoic acid (DEDA). These observations suggest for potential therapeutic efficacy of sPLA₂ inhibitor in Krabbe disease.

1. Introduction

Krabbe disease is a lethal, progressive, autosomal recessive, neuro-degenerative disorder with no cure. It is characterized by the progressive demyelination and presence of globoid cells and thus, also called the ‘globoid cell leukodystrophy’. Krabbe disease is caused by a genetic deficiency of lysosomal hydrolase, *galactosylceramide β-galactosidase* (*GALC*;

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E.C. 3.2.1.46) (Suzuki, 2003). *GALC* degrades galactosylceramide, a major component of myelin, and other terminal β -galactose-containing sphingolipids, including psychosine (D-galactosylsphingosine). Unlike other sphingolipid storage diseases, abnormal accumulation of the primary substrate of the deficient enzyme, galactosylceramide, does not occur in the central nervous system as it can also be degraded by *G_{MI} ganglioside β -galactosidase* (EC 3.2.1.23) (Kobayashi et al., 1985; Suzuki, 2003). However, psychosine is not hydrolyzed by *G_{MI} ganglioside β -galactosidase*, thus accumulates excessively in brain of Krabbe patients (Svennerholm et al., 1980; Vanier and Svennerholm, 1976). The increased psychosine levels in the neural tissue are believed to lead to widespread degeneration of oligodendrocytes (OLs) and to subsequent demyelination (Cleland and Kennedy, 1960).

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 (Schulte and Stoffel, 1993), in which it catalyzes the transfer of galactose to ceramide and sphingosine to form galactosylceramide and psychosine, respectively (Cleland and Kennedy, 1960; Mitsuo et al., 1989). Under normal conditions, psychosine in the brain is maintained at a very low level by the activities of *GALC*. However, psychosine continues to accumulate under *GALC* deficient conditions and may account for as much as 50% of cerebroside (Vanier and Svennerholm, 1976; Vanier and Svennerholm, 1975).

Myelination involves specific stagewise differentiation of proliferating OL precursor cells (OPCs) into post-mitotic OLs (Baumann and Pham-Dinh, 2001). Each differentiation stage is identified by morphological features and specific patterns of marker expression. 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-R α). 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'-phosphodiesterase) and synthesis of galactosylceramide. Mature-OLs extend myelin membranes and express myelin proteins such as proteolipid protein (PLP) and myelin basic protein (MBP) (Baumann and Pham-Dinh, 2001). 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 (Baumann and Pham-Dinh, 2001). The galactosylceramide can be converted to psychosine by deacylation under deficiency of *GALC* activity (Svennerholm et al., 1980). The *CGT* expression and thus enhanced *CGT* activity during this stage may also participate in increased synthesis of psychosine (Cleland and Kennedy, 1960; Mitsuo et al., 1989), 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.

Our laboratory has made contributions to the understanding of Krabbe disease pathobiology including mechanisms of psychosine induced proinflammatory response by astrocytes, involvement of secretory phospholipase A₂ (sPLA₂, EC 3.1.1.4) in psychosine-induced OL loss, and involvement of psychosine-induced dysregulations of peroxisomes and

plasmalogens synthesis in loss of OLs and myelin (Contreras et al., 2008; Giri et al., 2002; Giri et al., 2006; Haq et al., 2003; Haq et al., 2006; Jatana et al., 2002; Khan et al., 2005). However, most of the above mechanistic studies were based on use of exogenous psychosine supplement. In addition, we were unable to study the role of psychosine in OL differentiation using exogenous psychosine due to the detergent like property of exogenous psychosine (Suzuki, 1998). In this study, we investigated whether endogenous psychosine accumulation affects OL differentiation and survival by knock-down of *GALC* gene with RNA interference (RNAi) method. We report that the increase in *CGT* expression and thus increased synthesis and accumulation of psychosine in *GALC*-silenced differentiating OLs causes an impediment to the normal OL differentiation both by decreasing the expression of myelin lipids and protein and by inducing the cell death of maturing OLs.

2. Results

2-1. Knock-down of *GALC* induces psychosine accumulation in differentiating MO3.13 human OPC like cells

In the brain, *CGT* is primarily expressed in the differentiating and differentiated OLs (Schulte and Stoffel, 1993). Because *CGT* catalyzes the synthesis of both galactosylceramide and psychosine, the increased *CGT* expression during the OL differentiation processes may induce psychosine accumulation under the *GALC* deficient condition. In this study, we induced the differentiation of MO3.13 human OPC-like cells by incubating the cells in differentiation media (serum free condition with 0.1μM phorbol 12-myristate 13-acetate) and daily changes in the expression of PDGF-Rα, a marker for OPCs and pro-OLs, PLP, a marker for mature-OL, and *CGT* were analyzed by Western analysis. Fig. 1A shows that incubation of MO3.13 cells in differentiation media abolished the expression of PDGF-Rα on day 1 but increased the expression of PLP on day 2 and 3 (Fig. 1A). *CGT* expression on day 0 was very low but markedly increased on day 2 after incubation in differentiation media (Fig. 1A), suggesting that *CGT* expression is accompanied with MO3.13 cell differentiation

To generate *GALC* deficient OLs, the *GALC* gene in MO3.13 cells were silenced by RNAi methods as described in experimental procedure. Fig. 1B shows that transfection of MO3.13 cells with *GALC* RNAi expression lenti-virus (*GALC*-LV) successfully decreased *GALC* protein expression as observed by Western analysis. Following the transfection, the cells were incubated in differentiation media for 3 days and the cellular levels of psychosine were analyzed by LC/MS/MS as described previously (Bielawski et al., 2006; Whitfield et al., 2001). As shown in Fig. 1C, the cells silenced for *GALC* accumulated very high amount of endogenous psychosine following the differentiation, while the cells transfected with non-targeting scramble RNAi (*SCR*-LV) accumulated undetectable range of psychosine. In contrast to psychosine, however, there was no such increase in galactosylceramide by *GALC* knock-down.

2-2. Knock-down of *GALC* inhibits differentiation and survival of MO3.13 human OPC like cells

We next assessed whether the endogenous psychosine accumulation by *GALC* knock-down would induce apoptotic cell death in differentiating MO3.13 cells. As expected, we observed that there was no obvious difference in the cell morphology between in the cells treated with *SCR-LV* and *GALC-LV* before initiation of differentiation [see cells in 0-day in differentiation media (0-DIDM) in Fig. 2Ai]. In addition, there was no detectable apoptosis in *GALC-LV* treated cells at 0-DIDM as shown by TUNEL assay (Fig. 2B). However, the cells treated with *GALC-LV* underwent severe cell body atrophy following initiation of OL differentiation (Fig. 2A-i, see cells marked with yellow arrow head). It is of interest to note that the atrophy observed in *GALC-LV* treated cells was also observed in exogenous psychosine (10 μ M, Matreya, Inc., State College, PA) treated wild type cells (Fig. 2A-ii; see cells marked with red arrow heads). Moreover, the cells treated with *GALC-LV* also underwent apoptotic cell death on day 3 following differentiation while the cells treated with *SCR-LV* did not show any obvious apoptosis (Fig. 2B). Accordingly, we also observed that both the psychosine treatment and the *GALC* silencing caused the down-regulation of OL-survival signaling cascades, such as Akt and Erk (Pang et al., 2007; Zaka et al., 2005) (Fig. 2C).

Our previous studies suggest that psychosine-induced dysfunction of peroxisomes may play an important role in the OL pathogenesis of Krabbe disease (Haq et al., 2006). Therefore, we next examined the role of endogenously generated psychosine on peroxisomal pathogenesis. In MO3.13 cell culture, the silencing of *GALC* in differentiating OLs decreased the cellular expression of DHAP-AT, first rate limiting enzyme for the synthesis of plasmalogens (Fig. 2D-i) and PPAR α and PEX11 mRNA expression (Fig. 2D-ii). In addition to these peroxisomal proteins, silencing of *GALC* in MO3.13 cells also reduced PLP gene expression following the incubation of cells in differentiation media for 3 days (Fig. 2D-iii), suggesting the possibility that psychosine accumulation during the OL differentiation may inhibit OL maturation process by inhibiting peroxisomal function as well as by reduced expression of myelin components.

2-3. Knock-down of *GALC* inhibits differentiation of primary rat OPCs

MO3.13 cell line is a useful alternative for study of human OLs but probably produces some degree of cell line artifact. Therefore we further investigated the role of psychosine in rat primary OPCs. Transfection of rat primary OPCs with *GALC-LV* successfully decreased the *GALC* protein expression as shown in Fig. 3A. At OPC stage, *GALC* knock down did not affect peroxisomal gene expression as observed by no change in mRNA expression of PPAR α and DHAP-AT between the cells treated with *GALC-LV* and *SCR-LV* (Fig. 3B-i). In addition, at this stage there were no obvious differences in cell morphology of A2B2 positive cells (Fig. 3B-ii) and in expression of PDGF-R α (Fig. 3B-iii) between in *SCR-LV* and *GALC-LV* transfected cells. However, the mRNA expression of PPAR α and DHAP-AT was markedly decreased in the *GALC* silenced differentiating OLs (Fig. 3C-i). Similarly, mRNA expression for MBP was also decreased (Fig. 3C-i). 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 shown in light microscopy (panels *a* and *b* in Fig 3C-ii) and fluorescent microscopy of MBP stained cells (panels *c*, *d*, *e*, and *f* in Fig 3C-ii). Similar to MO3.13 cells, some of *GALC* silenced cells undergoing differentiation also underwent cell death as observed by fragmented cell bodies (see yellow arrow head in panel *f* of Fig 3C-ii). Taken together, the data from primary OL culture and MO3.13 cell culture indicate 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.

2-4. Knock-down of *GALC* inhibits differentiation of MO3.13 human OPC like cells

Previously, our laboratory reported that inhibition of sPLA₂ with pharmacological inhibitor 7,7-dimethyleicosadienoic acid (DEDA) was able to inhibit exogenous psychosine-induced cell death ((Giri et al., 2006) and Fig. 4A). To examine the role of sPLA₂ in OL differentiation and apoptotic cell death under *GALC* silenced conditions, the expression of OL differentiation marker (PLP) and apoptotic cell death (TUNEL staining) were investigated following the incubation of MO3.13 cells transfected with *SCR-LV* or *GALC-LV* in differentiation media containing vehicle or 10μM DEDA (Sigma-Aldrich). Similar to the data shown in Fig. 2, MO3.13 cells transfected with *GALC-LV* had increased apoptotic cell death as shown by TUNEL assay and decreased PLP expression in the absence of DEDA treatment (Fig. 4B). However, DEDA treatment reduced apoptotic cell death and increased PLP expression in *GALC* deficient cells (Fig. 4B), suggesting that sPLA₂ activation and thus generation of its metabolites may play a role in endogenous psychosine-induced pathology in OL differentiation.

3. Discussion

According to ‘psychosine hypothesis’ psychosine accumulation is the primary cause of the rapid degeneration of the myelin-forming cells and consequent demyelination that is seen in Krabbe disease (Miyatake and Suzuki, 1972; Suzuki, 2003). Based on the hypothesis, a number of studies assessed the role of psychosine in OL degeneration using exogenous psychosine supplement. However, the cellular response to exogenous psychosine might differ from the responses to endogenous psychosine for several reasons. Therefore, we generated *in vitro* cell culture model in which endogenous psychosine accumulates as a result of *GALC* silencing by RNAi methods. In this study we report that the increase in *CGT* expression and thus increased synthesis and accumulation of psychosine in *GALC*-silenced differentiating OLs causes an impediment to the normal OL differentiation both by decreasing the expression of myelin lipids and protein and by inducing the cell death of maturing OLs.

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 (Baumann and Pham-Dinh, 2001; Vanier and Svennerholm, 1976; Vanier and Svennerholm, 1975). Because *CGT* catalyzes the synthesis of both galactosylceramide and psychosine, we hypothesized that the increased

CGT expression and psychosine synthesis during the differentiation of *GALC*-deficient OLs may trigger Krabbe pathology by altering proper OL differentiation process. To test the hypothesis, we first investigated whether OL differentiation involves an enhanced *CGT* expression. We observed that differentiation of OPC-like MO3.13 cells, which predominantly expressed the PDGF-R α (a marker for OPCs and pro-OLs), induced the expression of *CGT* along with the expression PLP, a marker for mature OLs, indicating that OPC differentiation into OL accompanies the increase in *CGT* expression. Under these experimental conditions, the cells that have normal basal expression of *GALC* contain almost undetectable range of psychosine levels (Fig. 1C). However, the cells silenced for *GALC* accumulated extremely high levels of endogenous psychosine following the differentiation. In contrast to psychosine, however, there was no such increase in galactosylceramide levels (Fig. 1C) as observed in the brain of Krabbe affected individuals (Suzuki, 2003). Unlike other sphingolipid storage diseases, abnormal accumulation of the primary natural substrate of *GALC*, galactosylceramide, does not occur under *GALC*-silenced conditions as it can also be degraded by *G_{M1} ganglioside β -galactosidase* (EC 3.2.1.23) (Kobayashi et al., 1985; Suzuki, 2003). However, psychosine is not hydrolyzed by *G_{M1} ganglioside β -galactosidase*, thus resulting in its abnormal accumulation under *GALC* silenced conditions (Svennerholm et al., 1980; Vanier and Svennerholm, 1976).

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 (Suzuki, 1998). In addition, we previously reported that sub-threshold concentration of exogenous psychosine also induces OL death by inducing proapoptotic cell signaling cascades while down-regulating the cell survival signaling cascades (Giri et al., 2006; Jatana et al., 2002). Therefore, we next assessed whether the endogenous psychosine accumulation by *GALC* knock-down would induce apoptotic cell death in differentiating MO3.13 cells. As expected, there was no obvious difference in the cell morphology and survival between in normal cells and the cells silenced for *GALC* before initiation of differentiation (Fig. 2A and B). However, the cells silenced for *GALC* underwent severe cell body atrophy and apoptotic cell death following initiation of OL differentiation as observed in the cells treated with exogenous psychosine. Accordingly, the both the psychosine treatment and the *GALC* silencing caused the down-regulation of OL-survival signaling cascades, such as Akt and Erk (Pang et al., 2007; Zaka et al., 2005) (Fig. 2C). Similar to the effect on MO3.13 cells, *GALC* knock-down of primary cultured rat OPCs also induced the cell death following the differentiation (Fig. 3C), suggesting that cellular effects of endogenous psychosine accumulation on OL apoptosis appear to share some mechanisms with that of exogenous psychosine.

Fig. 2B shows that about 25% of cell population died through apoptosis as a result of knock-down of *GALC*. The rest of cell population might be a mixed population of functionally intact cells and the cells still undergo apoptosis. Under the same experimental conditions, we observed that the residual cell population expressed decreased levels of peroxisomal proteins as well as myelin components (Fig. 2D). These data suggest that the oligodendrocytes that survive from psychosine toxicity during differentiation process under the *GALC* deficient conditions may express reduced levels of myelin and peroxisomal proteins. The cell death

prior to oligodendrocyte differentiation may be a critical event leading to dysmyelination under *GALC* deficient conditions. However, twitcher and Krabbe brains express decreased but significant amount of myelin, indicating that some proportion of oligodendrocytes survive from psychosine toxicity. Therefore, the regulation of expression of myelin and peroxisomal components by survived oligodendrocytes should also be considered a potential targets for Krabbe disease.

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 (Contreras and Rapoport, 2002; Hajra and Das, 1996; Kovacs et al., 2002; Kovacs and Krisans, 2003; Sprecher, 2000). In addition, peroxisomes are the major site for hydrogen peroxide production by oxidases and its degradation by catalase (Schrader and Fahimi, 2006). We have previously reported that psychosine induces loss of peroxisomal functions in *in vivo* and *in vitro* cell cultures by inhibiting gene expression and transactivity of PPAR α , one of the transcription factors required for expression of peroxisomal protein genes, and following inhibition of peroxisomal protein expression, such as DHAP-AT and PEX11. These studies, therefore, suggest that psychosine-induced dysfunction of peroxisomes may play an important role in the OL pathogenesis of Krabbe disease (Haq et al., 2006). In this study, we observed that the silencing of *GALC* in differentiating MO3.13 cells decreased the cellular expression of peroxisomal proteins (i.e. DHAP-AT and PEX11) and peroxisome proliferator-activated receptor, PPAR α (Fig. 2D). In addition, silencing of *GALC* also reduced PLP gene expression during the differentiation process (Fig. 2D). Similar to the effect on MO3.13 cell line, *GALC* knock down also decreased the expression of peroxisomal proteins (i.e. PPAR α and DHAP-AT) and MBP in differentiated primary rat OLs (Fig. 3C), while it did not show any obvious effects on the expression of peroxisomal proteins and PDGF-R α in the cells at OPC stage (Fig. 3B). Although the mechanisms underlying the observed inhibitory role of psychosine in the regulation of peroxisomal gene expression and myelin protein are not known at present, these data strongly suggest that psychosine accumulation during the OL differentiation may inhibit OL maturation process by inhibiting peroxisomal function as well as by reduced expression of myelin components, such as PLP and MBP.

Previously, our laboratory reported increased lysophosphatidylcholine (LPC) levels in the brains of Krabbe patients and twitcher mice (Giri et al., 2006). LPC is a product of PLA $_2$ and has been implicated to induce demyelination (Hall, 1972). We reported that treatment of OLs with psychosine increases the levels of LPC and arachidonic acid via activating sPLA $_2$ and causes apoptotic cell death (Giri et al., 2006). Moreover, sPLA $_2$ inhibitor DEDA was able to inhibit exogenous psychosine-induced cell death ((Giri et al., 2006) and Fig. 4A). Similar to these data, we also observed in this study that DEDA treatment inhibited the increased apoptotic cell death and the decreased PLP expression in MO3.13 cells silenced for *GALC*, suggesting that sPLA $_2$ activation and thus generation of its metabolites may play a role in endogenous psychosine-induced pathology in OL differentiation.

In summary, these data provide evidence that the increase in *CGT* activity during the OL differentiation causes endogenous psychosine accumulation under *GALC* deficient Krabbe conditions. The increase in endogenous psychosine accumulation may impede the OL

maturation process by inhibiting the synthesis of myelin constituents and increases apoptotic cell death via sPLA₂-dependent mechanism.

4. Experimental Procedure

4-1. Cell culture and RNAi transfection

MO3.13 human OPC line were maintained in Dulbecco's modified eagle's medium (DMEM 4.5 g/L glucose) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1x Penicillin/Streptomycin (Invitrogen, Carlsbad, CA) at 37°C in 5% CO₂/95% air. At 60~70% confluency the cells were treated with lenti-viral particles expressing either *GALC* RNAi (*GALC*-LV; Santacruz Biotech. Inc., Santa Cruz, CA) or non-targeting scramble sequence (*SCR*-LV; *Non-silencing GI PZ lentiviral shRNA mir negative control*, Thermo Scientific, Rockford, IL) and incubated overnight. Following the incubation of cells in fresh media for 48hrs, the differentiation of these cells were initiated by incubation in serum free DMEM containing 0.1µM of phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, MO, USA).

Rat OPCs were isolated from primary cultured rat mixed glial cells as described before in our report (Paintlia et al., 2010). The isolated OPCs were plated on laminin-2 coated culture dishes or glass cover slips in DMEM media supplemented with N2 supplement (Invitrogen), B27 supplement (Invitrogen), recombinant platelet derived growth factor (PDGF)-AA (10 ng/mL) and fibroblast growth factor-2 proteins (10 ng/mL). The purified OPCs were then transfected with lenti-viral particles expressing either *GALC* RNAi (*GALC*-LV; Thermo Scientific) or *SCR*-LV and incubated overnight. Following the transfection, the OPC transfected with lentivirus were incubated in fresh Sato medium supplemented with 1% FBS and 10 ng/mL of recombinant ciliary neurotrophic factor (CNTF) protein for differentiation.

4-2. Quantification of psychosine and galactosyl-ceramide

Psychosine and galactosyl-ceramide was quantified by Liquid Chromatography-Mass Spectrometry/Mass system (LC/MS/MS) at University Lipidomics Core facility as reported previously (Contreras et al., 2008). For psychosine quantification, lipid extracts from control and experimental cell culture samples (Whitfield et al., 2001) were subjected to mass spectrometric analyses by mass spectrometer equipped with electrospray ion source (ESI) and high performance liquid chromatography as described previously (LC/MS/MS) (Bielawski et al., 2006; Whitfield et al., 2001). Psychosine is identified and quantified by m(mass)/z(charge) in relation to standard psychosine.

4-3. Western analysis

Western blot analysis were performed as described previously (Won et al., 2003) by using antibody against PDGF-Rα (Abcam Inc., Cambridge, MA, USA), CGT (Abcam), PLP (SantaCruz Biotech.), β-actin (SantaCruz Biotech.), *GALC* (Abcam), phospho-Akt (Ser473) (Cell Signaling Tech. Inc. Panvers, MA, USA) and phospho-p44/p42 Erk (Thr202/Tyr204) (Cell Signaling).

4-4. Quantitative real time PCR

Extraction and purification of total RNAs and quantitative real time PCR were performed as described before in our report (Paintlia et al., 2010). The following primer sets were used for this experiment; human PPAR α (forward; 5'-ACC GGA ACA AAT GCC AGT AC-3', reverse: 5'-TCA GAT CTT GGC ATT CTT CC-3'), rat PPAR α (forward; 5'-TCG GGA TGT CAC ACA ATG CAA TCC-3', reverse: 5'-CGT GTT CAC AGG TAA GGA TTT CTA CC-3'), human peroxisomal biogenesis factor 11, (PEX11: forward; 5'-CGA TCT CTG AAG CAG CAT C-3' and reverse, 5'-CCT CCA AGT CCA ATG ATG C-3'), human dihydroxyacetone-phosphate acyltransferase (DHAP-AT: forward; 5'-CAC AGA CAA AGG GAA TGC TG-3' and reverse, 5'-AAG TAG TCC TCT TCA TGC AAG-3'), rat DHAP-AT (forward; 5'-CAT CAT CCT CAC AGA CAA AGG G-3' and reverse, 5'-CTT CAT GCA AGA GGC ATT TGG A-3'), and GAPDH for human and rat (forward: 5'-ATG ACA TCA AGA AGG TGG TG-3', reverse: 5'-CAT ACC AGG AAA TGA GCT TG-3'). The quantities of each target gene expression were normalized to the corresponding GAPDH mRNA quantities in test samples.

4-5. Immunofluorescent cell staining, *in situ* TUNEL assay, and microscopy

MO3.13 cells and the primary rat OLs grown in chamber slides (Lab-Tek, Naperville, IL, USA) were fixed with 4% formaldehyde in PBS. For immunofluorescent cell staining, the fixed slides were blocked with a normal serum-PBS solution and incubated with primary antibody against PLP, DHAP-AT (Abcam), O1 and O4 oligodendrocyte markers (R&D System, Minneapolis, MN, USA), A2B5 (Abcam), or MBP (Santacruz) at 4°C overnight followed by washing and incubation with Alexa Fluor® 594 (red) or 488 (green)-conjugated secondary antibody. For staining of apoptotic cells, terminal transferase (TdT) dUTP nick end-labeling (TUNEL) staining was performed on the cells using the ApopTag Fluorescein *in situ* Apoptosis Detection Kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Briefly, cells were incubated with TdT and digoxigenin-labeled dUTP for 60 min at 37°C and were then incubated with fluorescein conjugated antibody against digoxigenin for 30 min at 25°C in the dark. All slides were mounted with Fluoromount-G (Electron Microscopy Sciences, Hatfield, PA, USA) containing 4'-6-Diamidino-2-phenylindole (DAPI) for staining of nuclei. Slides were analyzed by immunofluorescence microscopy (Olympus BX-60) with an Olympus digital camera (Optronics, Goleta, CA, USA) using a dual-band pass filter.

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Highlights

- Psychosine accumulates in differentiating oligodendrocytes silenced for GALC.
- Psychosine accumulation inhibits synthesis of myelin proteins and lipids.
- Psychosine accumulation increases cell death of differentiating oligodendrocytes.
- Psychosine accumulation inhibits terminal differentiation of oligodendrocytes.
- sPLA2 is involved in psychosine-inhibited oligodendrocyte differentiation.

Fig. 1. Knock-down of *GALC* induces psychosine accumulation in differentiating MO3.13 human OPC like cells

A. Following the initiation of MO3.13 cell differentiation by incubation in differentiation media (DMEM containing 0.1 μ M PMA), changes in expression of oligo-differentiation markers (PDGFR for OPCs and proOLs, CGT for immature and mature OLs, and PLP for mature OLs) were assayed by Western analysis. The cells were incubated up to 3 days in differentiation media (3-DIDM). **B.** MO3.13 cells were transfected with lenti-virus expressing *GALC* RNAi (*GALC*-LV) or non-targeting scramble sequence (*SCR*-LV), and cellular levels of *GALC* and β -actin for internal loading control were analyzed for confirmation of *GALC* knock down. **C.** Following the differentiation of *SCR*-LV or *GALC*-LV transfected MO3.13 cells, for 3 days (3-DIDM), the cellular levels of psychosine and galactosylceramide (GalCer) were analyzed by LC/MS/MS as described by experimental procedure.

Fig. 2. Knock-down of *GALC* inhibits differentiation and survival of MO3.13 human OPC like cells

A. MO3.13 cells transfected with lenti-virus expressing *GALC* RNAi (*GALC*-LV) or non-targeting scramble sequence (*SCR*-LV) were incubated for up to 3 days in differentiation media (0~3-DIDM) and the resultant change in cell morphology was assessed by fluorescent microscopy following the O4 staining (green) (**A-i**) or by phase contrast microscopy (**A-ii**). Wild type MO3.13 cells were also treated with exogenous psychosine (PSY; 10μM), and time course change in cell morphology was also assessed by phase contrast microscopy (**A-ii**). **B.** Time-course effect of *GALC* silencing on OL apoptosis was assessed by terminal deoxynucleotidyltransferase dUTP end labeling (TUNEL; green) assay following the initiation of *GALC*-LV or *SCR*-LV transfected MO3.13 cells. **C.** The effect of exogenous psychosine treatment and *GALC* silencing on the phosphorylation of Akt at Ser473 (pAkt) and p44/p42 Erk at Thr202/Tyr204 (pErk) was examined by Western analysis following the treatment of MO3.13 cells with exogenous psychosine (10μM) for 3hrs or *GALC*-LV for 3 days. **D.** The effect of *GALC* silencing on the expression of peroxisomal proteins, such as DHAP-AT (**D-i**; red) and PEX11 (**D-ii**), and PPARα(**D-ii**), and myelin protein PLP (**D-iii**; red) was analyzed by immunofluorescence staining (**D-i** and **iii**) and quantitative real-time PCR analysis (**D-ii**). DAPI (4', 6-diamidino-2-phenylindole; blue) was used for nuclear staining for immunofluorescence assay.

Fig. 3. Knock-down of *GALC* inhibits differentiation of primary rat OPCs

A. The cellular levels of *GALC* enzyme and β -actin (for internal loading control) were analyzed in the primary cultured rat OPCs transected with lenti-virus expressing *GALC* RNAi (*GALC*-LV) or non-targeting scramble sequence (*SCR*-LV). **B.** Under the same experimental conditions, the mRNA expression for PPAR α and DHAP-AT (**B-i**), OPC morphology (**B-ii**), and PDGFR α expression (**B-iii**) were also investigated by real time PCR, immunofluorescent microscopy of A2B2 stained cells, or Western analysis using specific antibody in the primary cultured rat OPCs transected with *GALC*-LV or *SCR*-LV. **C.** Following the initiation of *GALC*-LV or *SCR*-LV transfected OPCs for 4 days in differentiation media (see material and method section), the mRNA expression of PPAR α , DHAP-AT, and MBP was analyzed by quantitative real time PCR analysis (**C-i**). In addition, the morphology of differentiated OLs was also assessed by phase contrast light microscopy and immunofluorescence microscopy of MBP stained cells (**C-ii**). Hoechst was used for nuclear staining.

Fig. 4. Knock-down of *GALC* inhibits differentiation of MO3.13 human OPC like cells

A. MO3.13 cells were treated with exogenous psychosine (10 μ M) and/or sPLA2 inhibitor 7,7-dimethyl eicosadienoic acid (DEDA; 10 μ M) for 24hrs and the change in cell morphology was assessed by phase contrast microscopy. **B.** Following transfection of MO3.13 cells with *GALC*-LV or SCR-LV, the cells were incubated for 3 days in differentiation media and the induction of apoptotic cell death and the expression of myelin protein (PLP expression) were assessed by terminal deoxynucleotidyltransferase dUTP end labeling (TUNEL) assay and immunofluorescence microscopy. DAPI (4',6-diamidino-2-phenylindole) was used for nuclear staining.