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Effect of Vitamin D3 Intake on the Onset of Disease in Murine Model of Human Krabbe Disease

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Abstract

Low vitamin D level is a risk factor for various late-onset central nervous system (CNS) demyelinating disorders. Herein, we investigated whether vitamin D deficiency influences disease in *twitcher* mice ($GALC^{twi/twi}$; *twi*), a murine model of krabbe disease (KD), an inherited disorder caused by galactocerebrosidase (GALC) deficiency that leads to psychosine accumulation, oligodendrocyte (OL) loss and CNS demyelination. We found that *in situ* 1,25-dihydroxyvitamin D3 level was reduced with a parallel increase in the expression of inflammatory cytokines and vitamin D catabolizing enzymes in the KD and *twi* mice brains compared with age-matched controls. Pups maintained on lactating heterozygous ($GALC^{twi/+}$) mother's milk that were fed vitamin D3 supplement diet till weaning and later on vitamin D3 supplement diet had delayed the body weight loss and the development of disease in *twi* mice. It delayed the onset of tremors and locomotor disabilities that eventually impacted the lifespan of *twi* mice (50 ± 2 days). Accordingly, the expression of antioxidant enzymes was increased with delayed psychosine accumulation, lipid-peroxidation and inflammatory response that eventually protected CNS myelin and axonal integrity in *twi* mice. *In vitro* studies revealed that 1,25-dihydroxyvitamin D3 enhances antioxidant defenses in OLs deficient of GALC or incubated with psychosine. Together, these data provide first evidence that vitamin D deficiency affects disease development in *twi* mice and that vitamin D3 supplementation has potential to improve the efficacy of KD therapeutics.

Keywords

Krabbe disease; twitchier mouse; demyelinating disease; oligodendrocyte; psychosine

INTRODUCTION

Globoid cell leukodystrophy or Krabbe disease (KD) is an autosomal recessively inherited neurodegenerative disorder caused by mutation in β -galactosylceramidase (GALC) (EC 3.2.1.46), an enzyme responsible for the catabolism of myelin glycosphingolipids (Suzuki and Suzuki 1970; Wenger DA et al. 2001). GALC deficiency results in the abnormal accumulation of myelin component galactolipid, psychosine, which is a toxic metabolite to

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oligodendrocytes (OLs) and Schwann cells in KD patients (Nagara et al. 1986; Tanaka et al. 1989). It leads to the demyelination of the central and peripheral nervous systems during early developmental stages in KD patients (Kobayashi et al. 1988; Seitelberger 1981). The underlying mechanism of psychosine toxicity to OLs/Schwann cells is not understood yet. We and other earlier documented that psychosine causes OL apoptosis in KD brain (Haq et al. 2003; Jatana et al. 2002; Zaka and Wenger 2004). It was attributed to the elevated levels of oxidative stress as evidenced by the protection of OLs from psychosine toxicity by treatment with anti-oxidant, N-acetyl cysteine (NAC) *in vitro* (Haq et al. 2003; Khan et al. 2005). NAC based therapy has been documented to attenuate oxidative stress (Hawkins-Salsbury et al. 2012) in the brain of *twitcher* mouse ($GALC^{twi/twi}$; *twi*), an authentic murine model of KD (Duchen et al. 1980; Kobayashi et al. 1980). These observations provide evidence that psychosine induced oxidative stress is critical in KD pathogenesis.

Overall toxicity of psychosine to brain cells (Cho et al. 1997; Ida et al. 1990; Jatana et al. 2002) may be responsible for the delayed growth rate and development of KD patients and *twi* mice. Typical neurological signs i.e., tremors are usually accompanied with hind leg weakness after 20 days of age in *twi* mice that are followed by rapid disease progression and death by 40 days of age (De Gasperi et al. 2004; Suzuki and Taniike 1995). Hematopoietic stem cell transplantation therapy is reported to slow down KD progression in infants as well as the late-onset forms of KD in adults when administered before the onset of clinical symptoms (Escolar et al. 2005; Shapiro et al. 1995). Likewise, bone marrow transplantation is reported to provide positive outcomes in *trs* mouse model of KD (Luzi et al. 2005). Recently, an adeno-associated virus gene therapy demonstrated promise in *twi* mice (Rafi et al. 2012). The current focus of KD research is to search for better therapeutic agent to complement the existing therapeutics for KD management.

A large body of evidence suggests that low vitamin D level are a risk factor for various late-onset demyelinating CNS disorders (Evatt et al. 2008; Hayes 2000; Kragt et al. 2009). The active form of vitamin D3, 1,25 dihydroxyvitamin D3 [$1,25-(OH)_2D_3$; VDH] is reported to regulate inflammatory responses both in the peripheral and CNS compartments (Mayne et al. 2011). In addition, it attenuates oxidative stress to protect cells via induction of antioxidant mechanisms (Bao et al. 2008; Kutuzova and DeLuca 2007; Noyan et al. 2005; Wiseman 1993). Importantly, pro-inflammatory cytokines are reported to be elevated in the human KD and *twi* mice via activation of resident glial cells (Itoh et al. 2002; LeVine and Brown 1997). Recently, we documented that cholesterol lowering drug, lovastatin enhances the vitamin D homeostasis in the animal model of a late-onset CNS demyelinating diseases, multiple sclerosis (MS) (Paintlia et al. 2012). Vitamin D homeostasis is maintained by the enzymatic activities of 1α -hydroxylase (CYP27B1) and 24-hydroxylase (CYP24A1) in the kidney (Omdahl et al. 2002). Moreover, psychosine is reported to accumulate in the brain, spinal cord, liver and kidney of fetal KD and in *twi* mice (Ichioka et al. 1987; Ida et al. 1994). Based upon this information, we hypothesized that psychosine accumulation may affect vitamin D homeostasis in KD and *twi* mice. Therefore, we first analyzed the expression of vitamin D catabolizing enzymes in the KD and *twi* mice brains and investigated the effect of vitamin D3 intake in *twi* mice.

MATERIALS & METHODS

Chemicals and Reagents

Dulbecco's modified Eagle medium (DMEM; 4.5 g/L glucose), FBS, laminin-2 (meosin) were purchased from Life Technologies (Grand Island, NY). Recombinant platelet-derived growth factor (PDGF)-aa, basic fibroblast growth factor-2 (FGF-2), ciliary neurotrophic factor (CNTF) proteins were purchased from R&D Systems (Minneapolis, MN). Anti-myelin basic protein (MBP) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). Anti-CYP24A1 and anti-CYP27B1 antibodies were purchased from Abcam. Anti-A₂B₅ MicroBeads and MS columns were purchased from MACS, Miltenyi Biotec Inc. Rabbit anti-malondialdehyde (MDA) polyclonal antibodies were purchased from Cell Biolabs (San Diego, CA). ECL-detecting reagents and nitrocellulose membranes were purchased from Amersham Biosciences (Arlington Heights, IL).

Human Autopsy Brain specimen

Two frozen human infantile KD brain and two frozen normal age-matched controls brain tissues were obtained from Brain and Tissue Bank (university of Maryland at Baltimore, MD).

Animals

Heterozygote breeding pairs (*GALC*^{twi/+}) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and Washington State University (WASU). Mice were housed in the animal care facility at the Medical University of South Carolina (MUSC) throughout the experiment and were provided with food and water *ad libitum*. All the animal experiments were conducted in accordance with accepted standards of humane care, as outlined in the ethical guidelines and approved by the MUSC's Animal Ethics Committee. The genotype of the mice was determined after postnatal day (PND) 2 by PCR amplification with primers specific for the *GALC* mutation (Sakai et al. 1996). Experimental animals in this study were restricted to those surviving past weaning (PND 21).

1,25-(OH)₂D₃ (VDH) Analysis

The measurement of VDH in the brain tissues was performed by extraction methods published earlier (Spach and Hayes 2005). Briefly, tissues were extracted with chloroform-methanol-4% KCl in water (1:2:0.8 v/v) mixture to recover the vitamin D metabolites. The extracts were then vacuum dried, suspended in ethanol, diluted in sample buffer, and analyzed in duplicate for VDH using human & mouse 1,25-dihydroxyvitamin D₃ ELISA kits (MyBioSource, Inc, San Diego, CA).

RNA preparation, cDNA synthesis and quantitative RT-PCR (QRT-PCR) analysis

Cells or tissues were carefully processed for RNA isolation using TRIZOL reagent according to the manufacturer's protocol as described previously (Paintlia et al. 2005). Synthesis of single-stranded cDNA was carried out from total RNA using 'iScript cDNA synthesis' kit (Bio-Rad) and QRT-PCR was performed using iCycler iQ Real-Time PCR Detection System from BIORAD Laboratories. Primer sets (Table 1) used in the study were

designed using Primer Quest and purchased from IDT-DNA Technology (Coralville, IA, USA). IQTM SYBR Green supermix was purchased from BIO-RAD (Hercules CA). Thermal cycling conditions were as follows: activation of iTaqTM DNA polymerase at 95°C for 10 min, followed by 35 cycles of amplification at 95°C for 30 s and 58.5°C for 1 min. The specificity and detection methods for data analysis are as described earlier (Paintlia et al. 2008).

Vitamin D3 Treatment

Starting from PND 1-2 heterozygous (*GALC*^{twi/+}) lactating mothers were fed a commonly used cholecalciferol (vitamin D3) adjusted diet purchased from Teklad Lab Animal Diets, Harlan. The dietary level of vitamin D3 in this study was 16.45 IU/g and the intake was approximately 5,000 IU/kg body weight in adult mice. As the result pups received vitamin D3 as a continuous dose in mother's milk and then once weaned, pups were continued on the same diet until sacrificed. Untreated wild-type and *twi* mice were maintained on regular Harlan's control diet (vitamin D3 2.5 IU/g). Vitamin D intake was estimated by weighing chow consumption of lactating mice and groups of pups.

Lifespan and Locomotors assessment

The *twi* mice were allowed to live as long as it could and maintained humanely. Lifespan of the *twi* mice were recorded as the age in days on the date of death or sacrifice (Hawkins-Salsbury et al. 2012). Mice weight was recorded every day. In order to follow the course of disease development, the onset day of tremor was noted as well as walking ability was scored daily starting from PND 18. Walking ability was assessed according to the method described by (Pannuzzo et al. 2010) scoring was done as follows: 0, normal walking; 1, wobbly gait, but able to maintain upright posture; 2, weakness in hind limb characterized by drooping of hindquarters while walking; 3, struggling to walk with hind limbs with occasional dragging of hind limbs; 4, dragging of hind limbs with propulsion entirely by forelimbs.

Analysis of psychosine

Brain samples of mice were quick-frozen in liquid nitrogen, and stored at -70 °C until further analysis. Analysis of psychosine was performed on brain extracts (University Lipidomics Core facility) by Liquid Chromatography/Mass Spectrometry/Mass system (LC/MS/MS) as reported previously (Bielawski et al. 2006; Whitfield et al. 2001).

Neuropathological Evaluations

A series of the tissue sections of spinal cords and that of brains were generated and stained with hematoxylin and eosin (H & E), Luxol Fast Blue (LFB) and Bielschowsky silver to detect inflammatory infiltrates, demyelination, and axonal loss, respectively. Neuropathological data was quantified on 6–8 sections per mice. Sections were mounted and examined under a light microscope (Olympus BX-60, Goleta, CA) and images were captured with an Olympus digital video camera (Optronics; Goleta, CA) using a dual band pass filter using Adobe Photoshop 7 software. Nuclei were counted in sections (n=5/mouse) using image J software (NIH). Single-immunostaining of the neurofilaments in the brain and

spinal cord sections was performed using anti-rabbit NF200 antibody (abcam) using standard methods (Paintlia et al. 2005).

Determination of intracellular reduced GSH level

Reduced glutathione (GSH) levels were measured in brain homogenates using a reduced-GSH detection kit (Sigma) per product manual instructions. Data are presented as $\mu\text{mole/mg}$ of protein.

Western blot analysis

Total cell lysates were prepared in ice-cold lysis buffer (RIPA buffer, Thermo Scientific) and processed for Western blot analysis as described previously (Paintlia et al. 2005).

Cell culture and GALC knock-down

OL progenitor cells (OPCs) were isolated from primary cultures of mixed glial cells as described before (Paintlia et al. 2010). Purified 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 PDGF- $\alpha\alpha$ (10 ng/mL) and FGF-2 proteins (10 ng/mL). OPCs were then transfected with lenti-viral particles of GFP vector plasmid expressing either *GALC* RNAi (*GALC*-LV; Thermo Scientific) or control RNAi (*SCR* LV) and incubated overnight. Following the transfection, the OPC were incubated in fresh Sato medium supplemented with 1% FBS and 10 ng/mL of CNTF for differentiation. The differentiation of the OPCs after *GALC* knockdown or treatment with VDH was measured at 4 days *in vitro* by fluorescence and light microscopy. Likewise, OPC differentiation after treatment with psychosine (2.5 μM) in the presence/of absence of VDH was determined by using light microscopy.

Analysis of mitochondrial content using MitoTracker red and JC-1 staining

Mitochondria were labeled using the mitochondria-specific dye MitoTracker Red (Molecular Probes) according to manufacturer's protocol. The final dye concentration was 40 nmol/L, and the incubation time was 30 min prior to visualization. Fluorescent microscopy was performed on live cells, which were analyzed by immunofluorescence microscopy (Olympus BX-60) with an Olympus digital camera (Optonics; Goleta, CA) using a dual-band pass filter. For JC-1 staining, cells grown on glass cover-slips were treated with psychosine and incubated with JC-1 dye (JC-1 MMP assay kit; Cayman Chemical Company, Ann Arbor, MI, USA) in medium for 15–20 min at 37°C. Cells were then washed twice with PBS and mounted on glass slides with PBS/glycerol (50 : 50). Cells were photographed directly for J-aggregates at the settings used for Texas red (excitation/emission = 590/610 nm) and for monomers at the settings used to detect FITC (excitation/emission = 485/535 nm) using Fluorescence microscopy (Olympus BX-60). Images were captured and processed using Adobe Photoshop 7.0 software.

Statistical analysis

Using the Student's unpaired t-test and one-way multiple range ANOVA (Student-Newman-Keuls to compare all pairs of columns), *p* values were determined for the respective

experiment from three identical experiments using GraphPad Prism 3.0 software (GraphPad Software Inc. San Diego, CA). Lifespan curves were generated through the Kaplan–Meier method and analyzed with the log-rank test. The criterion for statistical significance was $P < 0.05$.

RESULTS

Inflammation and vitamin D homeostasis in the human KD and *twi* mice brains

Earlier, we and others documented that the expression of pro-inflammatory cytokines i.e., TNF- α was elevated in the CNS demyelination in *twi* mice (Haq et al. 2006; LeVine and Brown 1997; Wu et al. 2001). Inflammation has an impact on vitamin D homeostasis in neurodegenerative disease conditions as evidenced from the deregulation of *CYP24A1* gene, which encodes for an enzyme that degrades 1,25-(OH) $_2$ D $_3$ (VDH) (Omdahl et al. 2002; Paintlia et al. 2012). As expected, TNF- α transcripts level was elevated (~2-fold) in the human KD brain compared to age-matched controls (Fig. 1A). It was accompanied with reduced *in situ* level of VDH in the human KD brains compared with age-matched controls (Fig. 1B). Accordingly, the expression of CYP27B1 (mRNA) and CYP24A1 (mRNA and protein) was elevated (~3-fold) in the human KD brain compared with age-matched controls (Fig. 1C-E). Consistent with human KD brain data, TNF- α transcripts level was also elevated in the *twi* mice brains compared with age-matched controls (Fig. 1F). It was accompanied with reduced *in situ* VDH concentration and the elevated *CYP24A1* gene expression in the CNS of *twi* mice evidenced by mRNA and protein analyses as compared to age-matched wild-type mice (Fig. 1G-J). These data provide evidence that inflammation may affect vitamin D sufficiency in the human KD and *twi* mice brains via regulation of vitamin D catabolism.

Effect of increased vitamin D3 intake in *twi* mice

Earlier, higher vitamin D3 intake has been reported to confer protection in various late onset CNS demyelinating disorders (Evatt et al. 2008; Hayes 2000; Kragt et al. 2009). We hypothesized that high vitamin D3 intake should prevent disease development in *twi* mice. To address this, *twi* mice pups were maintained on the milk of the heterozygote (*GALC*^{twi/+}) lactating mothers fed vitamin D3 supplement diet followed by on vitamin D3 supplement diet after weaning (Fig. 2A). Interestingly, postnatal intake of vitamin D3 had significantly increased the average life span of *twi* mice compared with those were on regular control diet, i.e., PND 50 ± 2 days versus PND 34 ± 3 days (Fig. 2B). In addition, the onset of tremors in *twi* mice that usually starts at PND 23.4 ± 2.4 was delayed till PND 35.2 ± 2.2 by vitamin D3 intake (Fig. 2C). *Tw*i mice are reported to exhibit a progressive body weight loss by PND 20 ± 2 and that was delayed until PND 34 ± 2 by vitamin D3 intake (Fig. 2D). Accordingly, locomotor disabilities that are observed after PND 14 in *twi* mice were also delayed until PND 30 by high vitamin D3 intake (Fig. 2E). The observed wobbly gait movements start at PND 16 and the worsening of the hind limbs and death at PND 34 ± 2 in *twi* mice were also delayed by high vitamin D3 intake (Fig. 2E). Together, these data provide evidence that the postnatal intake of high vitamin D3 slows disease development in *twi* mice.

Vitamin D3 intake slows psychosine accumulation in the brain of *twi* mice

Usually, psychosine is undetectable in the normal brain, while GALC deficiency lead to the accumulation of psychosine due to its impaired metabolism in the *twi* mice, which starts at the earliest by PND 7 with exponential increase during their life span (Igisu and Suzuki 1984). The progressive accumulation of psychosine in the KD and *twi* mice brains is attributed to the the expression of UDP-galactosyltransferase in differentiating OLs (Morell and Radin 1969). Therefore, we next measured the level of psychosine in the *twi* and wild-type mice brains using LC/MS/MS as described previously (Bielawski et al. 2006; Whitfield et al. 2001). As shown in Fig. 3, psychosine level was significantly elevated in the *twi* mice brains as compared to wild-type counterparts at PND 35. Unexpectedly, the psychosine accumulation was slow in *twi* mice until PND 35 (30%) by vitamin D3 intake and that was eventually increased at PND 50, irrespective of vitamin D3 intake (data not shown). These data provide evidence that high vitamin D3 intake delays the psychosine accumulation rate in *twi* mice brains.

Vitamin D3 slows neurodegeneration process in the CNS of *twi* mice

To understand how does high vitamin D3 intake affect the psychosine accumulation, thereby the development of disease in *twi* mice, we analyzed the brain and spinal cord sections of *twi* and age-matched wild-type mice. At PND 35, the observed increased level of TNF- α transcripts in the brain and spinal cord tissues of *twi* mice compared with wild-type counterparts was attenuated by vitamin D3 intake (Fig. 4A-B). Blinded analyses of the spinal cord and brain tissue sections of *twi* mice demonstrated that the number of nuclei as an indicator of inflammation, was also reduced by vitamin D3 intake as compared to those were on regular control diet at PND 35 (Fig. 4C-D). Inflammation was apparent specifically in the white matter of the brain and that of the lateral and dorsal funiculi of the spinal cord of *twi* mice (not depicted). Accordingly, the MBP transcript level was also reduced in the brain and spinal cord tissues of *twi* mice compared with age matched wild-type counterparts and that was reversed by vitamin D3 intake at PND 35 (Fig. 4E-F). Consistent with RNA data, demyelination was evident in the corpus callosum (Fig. 5A) and cerebellum (Fig. 5B) regions of brain as well as in the spinal cords of *twi* mice (Fig. 6A) at PND 35. On the other hand, vitamin D3 intake reduced CNS demyelination as evident from myelin loss in the brain and spinal cord tissue sections of *twi* mice (Fig. 5A-B and 6A). Interestingly, myelin density was higher in the brain and spinal cord sections of *twi* mice supplemented vitamin D3 compared with those were on regular control diet, however, some of the regions showed low myelin density (Figs. 5A-B and 6A). CNS demyelination was also evident from the observed reduced levels of the MBP isoforms in the brain and spinal cord tissues of *twi* mice and that was attenuated by vitamin D3 intake (Fig. 6B). Neuronal degeneration was evident from silver impregnation of the cell body and axons in the brain and spinal cord sections of *twi* mice and that was also attenuated by vitamin D3 intake (Figs. 5A-B and 6A). Neurofilament staining further demonstrated that neuronal axons were degenerated in the white matter of the brain corpus callosum and spinal cords of *twi* mice compared with wild type counterparts and that were attenuated by vitamin D3 intake (Fig. 7). Together, these data imply that vitamin D3 intake limits CNS inflammation and neurodegeneration in *twi* mice.

Vitamin D3 enhances antioxidant mechanism in the CNS of *twi* mice

We earlier reported that psychosine mediated generation of reactive oxygen species is detrimental to OLs (Giri et al. 2008; Giri et al. 2006; Khan et al. 2005). Importantly, VDH is known to enhance antioxidant defenses in cells (Banakar et al. 2004; Bobilev et al. 2011). Therefore, to determine whether VDH enhances antioxidant mechanisms to limit CNS demyelination in *twi* mice, we measured lipid peroxidation in the brain and spinal cord tissues of *twi* mice using anti-MDA antibodies. Lipid peroxidation is reported to produce reactive aldehyde i.e., MDA protein adducts under oxidative stress conditions. Compared with age-matched wild-type control group, MDA protein adduct level was significantly elevated, while reduced-GSH levels were decreased in the brain and spinal cord tissues of *twi* mice and that was reversed by vitamin D3 intake (Fig. 8A-B). However, reduced-GSH level was comparable to wild-type controls in the brain tissues of *twi* mice supplemented vitamin D3 at PND 35 (Fig. 8B). In addition, manganese superoxide dismutase (SOD2) transcript levels were also reduced in the brain and spinal cord tissues of *twi* mice compared with wild-type counterparts and that were reversed by vitamin D3 intake (Fig. 8C-D). Together, these data imply that vitamin D3 intake counteracts lipid peroxidation in *twi* mice via induction of antioxidant defenses.

1,25-(OH)₂D₃ (VDH) enhances antioxidant defenses and protects OLs from psychosine toxicity

To understand the underlying molecular mechanism by which VDH enhances antioxidant defenses in the CNS of *twi* mice, we silenced *GALC* gene in OLs or exposed primary OLs to the cytotoxic psychosine concentration and treated with VDH. *GALC* gene was silenced using Lentivirus (LV) infection in primary OLs. As expected, *GALC* depleted OLs underwent severe cell body degeneration following initiation of their differentiation into myelin forming OLs and that was reduced by VDH treatment (Fig. 9A, upper panel). Green fluorescence protein (GFP) expression was used as a marker of transfection for visualization of LV infected OLs (Fig. 9A, upper panel). As expected, mitochondrial membrane permeability was lost in *GALC* deficient OLs as revealed by mitotracker red fluorescence analysis and that was attenuated by VDH treatment (Fig. 9A; middle panel). In addition, the number of *GALC* deficient OLs under differentiation conditions was significantly reduced compared with controls and that was blocked by VDH treatment (Fig. 9B). Accordingly, the MBP isoforms level was also reduced in *GALC* deficient OLs under differentiating conditions compared with controls and that was reversed by VDH treatment (Fig. 9C). Likewise, cytotoxic concentration of psychosine reduced the number of primary OLs and that was blocked by VDH treatment (Fig. 10A). To determine whether psychosine induces ROS in OLs, we next determined the mitochondrial membrane potential in OLs using JC-1 dye (lipophilic cation). There was fewer number of OLs and lesser red J-aggregate fluorescence exposed to psychosine and that was blocked by VDH treatment (Fig. 10B). Together, these data imply that psychosine mediated generation of ROS affects mitochondrial health in OLs and that was attenuated by VDH.

DISCUSSION

The present study indicates that vitamin D deficiency, in part, contributes to human KD pathogenesis as evidenced by the postnatal intake of vitamin D3 had delayed the development of neurological disabilities and improved lifespan of *twi* mice. A large body of evidence suggests that vitamin D insufficiency is critical for various late-onset CNS demyelinating diseases (Evatt et al. 2008; Hayes 2000; Kragt et al. 2009). Interestingly, we observed increased vitamin D catabolism in the brains of human KD and *twi* mice as evidenced by increased *in situ* expression of the vitamin D catabolizing enzymes and the reduced VDH levels (Fig. 1A-J). The supplementation of vitamin D3 *twi* mice pups through milk till weaning age and then vitamin D3 supplement diet had delayed the appearance of neurological disabilities and improved their lifespan. It was accompanied with the delayed onset of locomotor dysfunction, gait behavior and the loss of body weight in *twi* mice (Fig. 2A-E). In addition, vitamin D intake delayed the onset of tremors and twitching in *twi* mice compared with those on regular control diet. These beneficial effects of vitamin D3 intake were ascribed to the delayed accumulation of psychosine, inflammation and neurodegeneration the CNS of *twi* mice (Figs. 3-7). Further examination of the brain and spinal cord tissue revealed that vitamin D intake reduces the generation of ROS and enhances antioxidant defenses in *twi* mice. In line with this, *in vitro* studies demonstrated that VDH protects mitochondrial health in OLs deficient of GALC or primary OLs exposed to cytotoxic concentration of psychosine (Figs. 9-10). Altogether, these data provide first evidence that vitamin D3 intake can delay neurological impairments in human KD patients.

Human KD is a progressive CNS disease caused by the deficiency of lysosomal enzyme, GALC which is responsible for the degradation of specific galactolipids, including psychosine, galactocerebroside and sulfatides that are important in the production and maintenance of myelin (Wenger et al. 1997). In the absence of GALC enzyme, psychosine degradation is impaired, which leads to the OL degeneration as well as the impaired generation of myelin forming OLs (Suzuki 1998; Zaka and Wenger 2004). Presently, hematopoietic stem cell transplantation is considered to be a successful therapeutic approach to slow down the disease progression in KD patients (Escobar et al. 2005; Shapiro et al. 1995). Human KD is represented by *twi* mouse that develops sickness after ~21 days of age leading to steady weight loss, muscle weakness, and hind-leg paralysis. *Tw*i mouse becomes hunched by 30 days and usually die before 40 days of age (Wenger et al. 1997). Several treatment trials are conducted in *twi* mice including bone marrow transplantation (BMT) (Hoogerbrugge et al. 1988; Luzi et al. 2005), stem cell transplantation (Neri et al. 2011; Ripoll et al. 2011), *in vivo* and *ex vivo* gene therapies (Galbiati et al. 2009; Lin et al. 2007; Lin et al. 2005), substrate reduction therapy (Biswas and LeVine 2002), enzyme replacement therapy (Lee et al. 2005), and lately small molecule therapies (Lee et al. 2010; Luzi et al. 2009). BMT has been so far the most effective therapeutic strategy in the treatment of *twi* mice that prolongs their life from ~40 days to an average of 80 days. However, the administration of *GALC* gene through adeno-associated virus demonstrated the prolonged lifespan of *twi* mice up to 8 months (Rafi et al. 2012). Our findings demonstrate that the postnatal intake of vitamin D3 provides beneficial effect and slows the disease outcomes and enhances the lifespan up to 50 ± 2 days in *twi* mice. These protective

effects of vitamin D3 in *twi* pups could be attributed the lactating mother's milk as high-dose maternal supplementation of vitamin D3 prevented hypovitaminosis D in the mothers and the nursing infants (Hollis and Wagner 2004).

Similar to various late-onset demyelinating CNS diseases (34-36), our findings provide first evidence that vitamin D deficiency, in part, contributes to KD pathogenesis. Inflammation is critical for vitamin D insufficiency as evident from the observed hypovitaminosis D due to the deregulation of CYP24A1 expression in the immune cells in the animal model of MS (Paintlia et al. 2012). In agreement with this, we observed the increased expressions of TNF- α and that of CYP24A1 and CYP27B1 enzymes that could be responsible for *in situ* VDH depletion in the human KD and *twi* mice brains (Fig. 1A-J). In line with this, we recently documented the retardation of postnatal bone growth in *twi* mice (Contreras et al. 2010) and that, in part, could be associated with VDH insufficiency. In the CNS, it is unclear which brain cell type contributes to hypovitaminosis D in human KD and *twi* mice brains. We hypothesize that activated astrocytes may be expressing a higher level of CYP24A1 and CYP27B1 in the CNS of human KD and *twi* mice as activated astrocytes are reported to express a higher level of the CYP24A1 in the MS brain lesions (Smolders et al. 2013). VDH is reported to enhance cellular antioxidant defenses (Bobilev et al. 2011). We observed the attenuation of lipid peroxidation and the preservation of the reduced-GSH level in the CNS of *twi* mice by vitamin D3 intake, suggesting that vitamin D3 may be enhancing antioxidant defense in *twi* mice. In support of this, we earlier documented that antioxidant based therapy holds promise for human KD (Khan et al. 2005). In addition, N-acetyl cysteine treatment was found to be effective to improve bone marrow transplantation in *twi* mice (Hawkins-Salsbury et al. 2012).

The exact mechanism behind the observed beneficial effects of vitamin D3 intake on the prolonged life span of *twi* mice is not understood, at present. Cytokines secreted by activated glial cells are reported to contribute to the pathogenesis of demyelinating lesions in the *twi* mice (Giri et al. 2002; Wu et al. 2001). TNF- α expression was elevated in the *twi* mouse brain and that was attenuated by vitamin D3 supplementation (Fig. 4A-B). In addition to inflammatory mediators, psychosine is reported to be detrimental to OLs which induces their apoptotic cell death (Haq et al. 2006). These findings suggest that inflammation and the subsequent loss of OLs intensify the accumulation of psychosine due to generation of myelinating OLs in *twi* mice. However, anti-inflammatory and anti-oxidative activities of VDH may delay the accumulation rate of psychosine by reducing inflammation and the protection of OLs (Fig. 3). In agreement with these, lipid peroxidation as evident from the increased MDA protein adducts with corresponding reduction of the reduced-GSH level and SOD2 expression in the CNS of the *twi* mice were blocked by vitamin D3 intake (Fig. 8A-D). In vitro studies revealed that VDH protects mitochondrial health in *GALC* deficient OLs or in OLs exposed to the cytotoxic concentration of psychosine (Fig. 9 and 10). VDH is reported to enhance cellular antioxidant defense via attenuation of MDA level and induction of GSH synthesis (Banakar et al. 2004). In addition, VDH is reported to provide anti-inflammatory effects in different disease animal models (Pedersen et al. 2007; Sloka et al. 2011). Despite the fact that vitamin D3 could not be expected to cure an inborn metabolic defect, we hypothesize that it might have a beneficial effect to minimize cytopathic effect of

psychosine in the KD brain. Importantly, anti-inflammatory and antioxidant activities of VDH contributes to reduce clinical disabilities and prolong the lifespan of *twi* mice. Detailed studies about how does VDH protect OLs from psychosine toxicity in *twi* mice are currently underway.

CONCLUSIONS

Our findings provide unprecedented evidence that vitamin D deficiency is contributing to the KD pathogenesis as its exogenous supplementation delays the neurological disabilities and improves the lifespan of *twi* mice. It deserves further investigations to evaluate the efficacy of vitamin D3 as stand alone therapy or adjunct therapy in combination with currently ongoing enzyme or stem cell based therapies for the management of human KD.

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HIGHLIGHTS

- Vitamin D deficiency is a risk factor for CNS demyelinating disorders.
- Vitamin D deficiency contributes to KD pathogenesis.
- Vitamin D3 treatment delays the tremors and enhances the life span of *twitcher* mice.
- Vitamin D3 therapy enhances antioxidant defenses in the CNS of *twitcher* mice.
- Antioxidative activity of vitamin D hormone protects OLs from psychosine toxicity.

Fig. 1. Vitamin D deficiency in *twitcher* (*GALC^{twi/twi}, twi*) mice and human KD brain
 Human KD and age-matched control (CON) brain slices were cut from the white matter area of the cerebellum. Lactating heterozygous (*GALC^{twi/+}*) mothers with *twi* mice pups were maintained on a regular control diet and the brain and spinal cord samples of wild-type (WT) and *twi* mice were collected at PND 35. The average of two samples analyzed in triplicate depicts a change in TNF-α expression (A), 1,25(OH)₂D₃ level (B), CYP27B1 expression (C) and CYP24A1 expression (D) in the human brain samples. E, representative immunoblot depicts the levels of CYP24A1 and β-actin proteins in the brain samples. The composite mean ± S.E. of three or four samples analyzed in triplicate depicts a change in TNF-α expression (F), 1,25(OH)₂D₃ level (G), CYP27B1 expression (H) and CYP24A1 expression (I) in the mouse brain samples. J, representative immunoblot depicts the levels of CYP24A1 and β-actin proteins in the mouse brain samples. Statistically significant as indicated: *** P<0.001.

Fig. 2. Vitamin D3 supplement diet lessens neurological disabilities in *twi* mice

Lactating heterozygous ($GALC^{twi/+}$) mothers and the weaned *twi* mice were maintained on regular control or vitamin D3 supplement diet as depicted (A). B, The lifespan improvement of *twi* mice by vitamin D3 intake (n=12). The composite mean $\pm$ S.E. of 6-8 mice with age-matched wild-type (WT) and *twi* mice depict (B), onset of tremors (C), body weight management (D) and walking ability (E). Statistically significant as indicated: ** $P < 0.01$ and *** $P < 0.001$.

Fig. 3. Vitamin D3 slows psychosine accumulation in the *twi* mouse brain

The composite mean $\pm$ S.E. of the 6 mice depict a level of psychosine in the age-matched WT and *Twi* mice brains at PND 35. Statistically significant as indicated: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Fig. 4. Vitamin D3 supplement diet attenuates inflammation and myelin protein loss in the CNS of *twi* mice

A and B, the composite mean $\pm$ S.E. Of 6-8 mice depict a level of TNF- α transcripts in the brain and spinal cord samples of the age-matched WT and *twi* mice. C and D, the composite mean $\pm$ S.E. of 6-8 mice depict number of nuclei/field of the brain and spinal cord sections of the age-matched wild-type and *twi* mice. E and F, the composite mean $\pm$ S.E. of 6-8 mice depict a level of MBP transcripts in the brain and spinal cord samples of the age-matched WT and *twi* mice. Statistically significant as indicated: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, NS; non-significant.

Fig. 5. Vitamin D3 supplement diet attenuates neurodegeneration in *twi* mice

A representative field depicts white matter morphology in the brain sections stained with H&E plus LFB and Bielschowsky silver stains. Asterisk depicts demyelination and axonal loss.

Fig. 6. Vitamin D3 supplement diet attenuates neurodegeneration in *twi* mice

A representative field depicts white matter morphology in the spinal cord sections stained with H&E plus LFB and Bielchowsky silver stains. B, representative immunoblots depicts a MBP level in the brain and the spinal cord of mice. Asterisk depicts demyelination and axonal loss.

Fig. 7. Vitamin D3 supplement diet attenuates neurodegeneration in *twi* mice

A representative field depicts white matter morphology in the brain and spinal cord sections immunostained with anti-NF-200 antibodies. Asterisk depicts demyelination and axonal loss.

Fig. 8. Vitamin D3 slows psychosine accumulation and enhances antioxidant mechanisms in the *twi* mouse brain

The composite mean $\pm$ S.E. of the 6 mice depict a level of reduced-GSH (B) in the age-matched WT and *Tw* mice brains at PND 35. A, representative immunoblots depict the level of MDA protein adducts in the brain and spinal cord samples. C and E, the composite mean $\pm$ S.E. of 6-8 mice depict the levels of SOD2 transcripts in the brain and spinal cord samples at PND 35. Statistically significant as indicated: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Fig. 9. VDH protects OLs deficient of GALC

OLs (2,000 cells/cm²) cultured in 6-well cell culture plates or glass chamber slides were incubated with *GALC*-LV or *SCR*-LV particles and treated with VDH (100 nM) as detailed under materials and methods. A, representative field of the slides depicts GFP expression (upper panel), the morphology of mitochondria (middle panel) and cells by phase contrast (lower panel) in OLs after 4 days of differentiation in the presence or absence of VDH. B, the composite mean $\pm$ S.E. of 6 or 8 representative fields of 5 slides analyzed depicts the number of OLs. C, the representative immunoblots depicts a MBP level in the OLs after treatment with VDH. Statistically significant as indicated: *** $P < 0.001$.

Fig. 10. VDH protects mitochondrial membrane potential in OLs exposed to psychosine OLs (2,000 cells/cm²) cultured in glass chamber slides were exposed to psychosine (2.5 μ M). A, the composite mean $\pm$ S.E. of 6 representative fields of 5 slides analyzed depicts the number of OLs exposed to psychosine in the presence or absence of VDH (100 nM) for 4 days *in vitro*. B, Representative field of the slides depicts JC-1 staining of OLs exposed to psychosine in the presence or absence of VDH for 8 h. Arrowhead (yellow) depicts unhealthy cells and arrowhead (red) depicts healthy cells. Statistically significant as indicated: *** P<0.001.

Table 1

List of primers used for quantitative real-time PCR analysis

Gene Name	Primer Sequence
Mouse	
β -actin	FP: 5'-CCGTAAAGACCTCTATGCCAAC -3'; RP: 5'-AGGAGCCAGAGCAGTAATCT-3'
TNF- α	FP: 5'-CATCTTCTCAAAATTCGAGTGACAA -3'; RP: 5'-TGGGAGTAGACAAGGTACAACCC-3'
CYP24A1	FP: 5'-CTAAAGGAGTCCATGAGGCTTAC -3'; RP: 5'-TCCTTTGGGTAGCGTGATTTC -3'
MBP	FP: 5'-ATTGTGACACCTCGAACACCACCT -3'; RP: 5'-ATTAAGAAGCCGAGGGCAGGATTTC-3'
SOD2	FP: 5'-AGGCTGTGAATGGAGTTCAGTGGT -3'; RP: 5'-TAAGCACTCCTCAAGCAGGAGCAA -3'
Human	
β -actin	FP: 5'-TCCACCGCAAATGCTTCT-3'; RP: 5'-AGCCATGCCAATCTCATCTT-3'
TNF- α	FP: 5'-CAGCTTTGATCCCTGACATCT-3'; RP: 5'-TCTAGGTGAGGTCTTCTCAAG-3'
CYP24A1	FP: 5'-CAGGGTTCAGTCTGGTTTA-3'; RP: 5'-TCATGGAAGATGCAAGACAGAA-3'
CYP27B1	FP: 5'-GGCCCAGATCCTAACACATTT-3'; RP: 5'-ACTGTAGGTTGATGCTCCTTTC-3'

Note; FP stands for forward primer and RP stands for reverse primer.