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Ceramide-Mediated Insulin Resistance and Impairment of Cognitive-Motor Functions

Suzanne M. de la Monte^{a,b,*}, Ming Tong^a, VanAnh Nguyen^a, Mashiko Setshedi^b, Lisa Longato^a, and Jack R. Wands^a

^aDepartments of Pathology (Neuropathology), Neurology, and Medicine, and the Liver Research Laboratory, Rhode Island Hospital, Providence, RI, USA

^bWarren Alpert Medical School of Brown University, Providence, RI, USA

Abstract

Obesity, type 2 diabetes mellitus (T2DM), and non-alcoholic steatohepatitis (NASH) are associated with cognitive impairment, brain insulin resistance, and neurodegeneration. Recent studies linked these effects to increased pro-ceramide gene expression in liver and increased ceramide levels in serum. Since ceramides are neurotoxic and cause insulin resistance, we directly examined the role of ceramides as mediators of impaired signaling and central nervous system function using an *in vivo* model. Long Evans rat pups were administered C2Cer:N-acetylsphinganine or its inactive dihydroceramide analog (C2DCer) by i.p. injection. Rats were subjected to rotarod and Morris water maze tests of motor and cognitive function, and livers and brains were examined for histopathology and integrity of insulin/IGF signaling. C2Cer treatment caused hyperglycemia, hyperlipidemia, and mild steatohepatitis, reduced brain lipid content, and increased ceramide levels in liver, brain, and serum. Quantitative RT-PCR analysis revealed significant alterations in expression of several genes needed for insulin and IGF-I signaling, and multiplex ELISAs demonstrated inhibition of signaling through the insulin or IGF-1 receptors, IRS-1, and Akt in both liver and brain. Ultimately, the toxic ceramides generated in peripheral sources such as liver or adipose tissue caused sustained impairments in neuro-cognitive function and insulin/IGF signaling needed for neuronal survival, plasticity, and myelin maintenance in the brain. These findings support our hypothesis that a liver/peripheral tissue-brain axis of neurodegeneration, effectuated by increased toxic lipid/ceramide production and transport across the blood-brain barrier, could mediate cognitive impairment in T2DM and NASH.

Keywords

Central nervous system; ceramide; diabetes mellitus; insulin resistance; neurodegeneration; neurons; non-alcoholic; steatohepatitis

INTRODUCTION

Obesity, type 2 diabetes mellitus (T2DM), nonalcoholic steatohepatitis (NASH), Alzheimer's disease (AD), and experimental AD-type neurodegeneration produced by intra-cerebral

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*Correspondence to: Dr. Suzanne M. de la Monte, MD, MPH, Pierre Galletti Research Building, Rhode Island Hospital, 55 Claverick Street, Room 419, Providence, Rhode Island 02903, USA. Tel.: +1 401 444 7364; Fax: +1 401 444 2939; Suzanne_DeLaMonte_MD@Brown.edu.

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streptozotocin treatment are all associated with insulin resistance, oxidative stress, mitochondrial dysfunction, and pro-inflammatory cytokine activation [1–9]. Increasing evidence suggests that ceramides play a major role in the pathogenesis of obesity, T2DM, and NASH [10–13] because ceramides cause insulin resistance [14–18] and they activate proinflammatory cytokines. On the other hand, ceramide synthesis is stimulated by pro-inflammatory cytokines, such as tumor necrosis factor- α (TNF- α) [13], and pro-inflammatory cytokines are highly activated in obesity, T2DM, NASH, and AD [19–25], and previous studies demonstrated that ceramide levels are increased in AD brains [12,26]. We hypothesize that ceramides mediate some aspects of brain insulin resistance associated with both AD and T2DM/obesity mediated neurodegeneration because ceramides: 1) can be generated in brain [10,13,27,28]; 2) are increased in various dementia-associated diseases, including AD [10,12,29,30]; and 3) are lipid soluble and therefore likely to readily cross the blood-brain barrier, providing a mechanism by which obesity, T2DM, or NASH could lead to brain insulin resistance.

Ceramides represent a family of lipids generated from fatty acid and sphingosine [13,31,32]. Ceramides are distributed in cell membranes, and in addition to structural functions, they regulate signaling pathways that mediate growth, proliferation, motility, adhesion, differentiation, senescence, and apoptosis. Ceramides are generated biosynthetically through ceramide synthase and serine palmitoyltransferase activities [27,28,33]. Alternatively, ceramides are generated by sphingolipid catabolism through activation of neutral or acidic sphingomyelinases [28,32], or degradation of complex sphingolipids and glycosphingolipids localized in late endosomes and lysosomes [31]. Ceramides are metabolized to sphingosine by ceramidases, and ceramide, sphingosine, and sphingosine-1-phosphate are implicated in the pathogenesis of obesity and insulin resistance [28]. Correspondingly, inhibition of ceramide synthesis or its accumulation prevents obesity-associated insulin resistance [14,17].

Complex sphingolipids including gangliosides [34], and long-chain naturally occurring ceramides (i.e., up to 24 carbon atoms in length) [35], stimulate cell growth and functions, whereas sphingosine-containing lipids, including shorter ceramides, have inhibitory effects, resulting in increased apoptosis and cytotoxicity, or impaired growth [34,36,37]. Sphingomyelinases are activated by pro-inflammatory cytokines (i.e., TNF- α [13]), and pro-apoptotic stimuli including ionizing radiation, Fas, and trophic factor withdrawal [31,32]. Ceramides impair cellular functions and cause apoptosis by: 1) modulating the phosphorylation states of various protein, including those that regulate insulin signaling [38]; 2) activating enzymes such as interleukin-1 β converting enzyme (ICE)-like proteases, which promote apoptosis [31]; or 3) inhibiting Akt phosphorylation and kinase activity [39] through activation of protein phosphatase 2A [40].

In obesity, adipose tissue, skeletal muscle, and liver tissue exhibit abnormal sphingolipid metabolism that results in increased ceramide production, inflammation, and activation of pro-inflammatory cytokines, and impairments in glucose homeostasis and insulin responsiveness [13,16,28]. In both humans with NASH [41], and the C57BL/6 mouse model of diet-induced obesity with T2DM and NASH [42], ceramide levels in adipose tissue are elevated due to increased activation of sphingomyelin transferase, and acidic and neutral sphingomyelinases [31]. In addition, ceramide synthase and sphingomyelin transferase mRNA levels in liver are increased during the early stages of hepatic steatosis, but with emergence of NASH and neurodegeneration, those mRNA transcripts decline while sphingomyelinase gene expression increases [43]. Since the neurodegeneration in diet-induced obesity was not associated with increased central nervous system (CNS) expression of pro-ceramide genes, we extended the analysis by directly investigating the role of exogenous ceramide exposure in the pathogenesis of neurodegeneration and brain insulin resistance using an *in vivo* model.

MATERIALS AND METHODS

Materials

Ceramide analogs, D-erythro-Ceramide (C2Cer: N-acetyl-D-erythro-sphingosine, C6Cer: N-Hexanoyl-D-erythro-Sphingosine), dihydroceramide analog (C2D Cer; Dihydro-N-Acetyl-D-erythro-Sphingosine) were purchased from CalBiochem (San Diego, CA). Histochoice fixative was purchased from Amresco, Inc. (Solon, OH). The Amplex UltraRed soluble fluorophore, and the Akt Pathway Total and Phospho 7-Plex Panels were purchased from Invitrogen (Carlsbad, CA). MaxiSorb 96-well plates used for ELISAs were from Nunc (Thermo Fisher Scientific; Rochester, NY). QIAzol Lysis Reagent for RNA extraction and QuantiTect SYBR Green PCR Mix were obtained from Qiagen, Inc (Valencia, CA). The AMV 1st Strand cDNA Synthesis kit and Universal Probe Library and rat β -actin reference gene assay were purchased from Roche Applied Science (Indianapolis, IN). Monoclonal anti-ceramide, polyclonal anti-phospho-Tau ($p^{S199/202}$ -Tau), and Tau, and synthetic oligonucleotides used in quantitative polymerase chain reaction (qPCR) assays were purchased from Sigma-Aldrich Co. (St. Louis, MO). Fine chemicals were purchased from CalBiochem (Carlsbad, CA) or Sigma-Aldrich (St. Louis, MO). The triglyceride assay kit was from Sigma (St. Louis, MO).

Experimental model

Long Evans rat pups were given 7 alternate day intraperitoneal (i.p.) injections of 2.0 mg/kg (50 μ l volumes) ceramide analogs, D-erythro-N-Acetyl-Sphingosine (C2Cer) or D-erythro-Dihydro-N-Acetyl-Sphingosine (C2 Dihydroceramide; C2D), beginning on postnatal day 3. C2D is a structurally similar, inactive analog of C2Cer, and was used as a negative control. The doses of C2Cer and C2DCer were within the ranges employed to generate other *in vivo* models [44–50], and the concentrations we used previously to demonstrate ceramide-mediated neuronal insulin resistance *in vitro* [51]. In addition, we performed empirical studies to assess the dose range and time course of treatment that were not acutely toxic, yet caused peripheral insulin resistance. Ceramide reagents were dissolved in ethanol and diluted in sterile saline prior to use. All animals survived the procedure, and did not exhibit any aberrant behavior or adverse responses such as failure to thrive, poor grooming, reduced physical activity, or weight loss. Rats were weighed weekly. Rats were subjected to rotarod testing on P15–P16, and Morris water maze testing on P24–P28. On P30, after an overnight fast (14 h), rats were sacrificed by i.p. injection of 120 mg/kg pentobarbital, and blood, liver, and brain were harvested.

Blood or serum was used to measure glucose, insulin, neutral lipid, and ceramide levels as previously described [43,52]. Brain glucose levels were measured in PBS homogenates of temporal lobe tissue using a glucometer and results were normalized to sample protein concentration (μ g/mg protein). Cerebella, temporal lobes, and liver were harvested for histopathological, biochemical, and molecular studies. For histopathology, tissue samples were immersion fixed in Histochoice and embedded in paraffin. Histological sections of brain (8- μ m thick) were stained with Luxol Fast Blue, Hematoxylin, and Eosin (LHE), while liver sections were stained with H&E. For molecular and biochemical assays, brain and liver tissues were snap-frozen in a dry ice-methanol bath and stored at -80°C . We studied cerebella and temporal lobes because both brain regions: 1) require intact insulin/IGF signaling to maintain their structural and functional integrity [53,54]; and 2) they are targets of neurodegeneration in insulin-resistance diseases [8,55–58]. Our experimental protocol was approved by the Institutional Animal Care and Use Committee at Lifespan-Rhode Island Hospital, and conforms to the guidelines set by the National Institutes of Health.

Rotarod testing

We used rotarod testing to assess long-term effects on motor function [59] resulting from the i.p. ceramide treatments. On P15, rats were trained to remain balanced on the rotating

Rotamex-5 apparatus (Columbus Instruments) at 1–4 rpm. On P16, rats ($n = 8–10$ per group) were administered 10 trials at incremental speeds up to 4 rpm, with 10 min rest between each trial. The latency to fall was automatically detected and recorded with photocells placed over the rod. However, trials were stopped after 30 s to avoid exercise fatigue. Data from trials 1–3 (1–2 rpm), 4–7 (2.5–3.5 rpm), and 8–10 (4 rpm) were culled and analyzed using the Mann-Whitney test.

Morris water maze testing

Morris water maze testing [60] of spatial learning and memory was performed on 4 consecutive days as previously described [3,4]. On the first day of testing, the rats were oriented to the water maze and educated about the location of the platform. On the 3 subsequent days of testing, the platform was submerged just below the surface, and rats were tested for learning and memory by measuring the latency period required to reach and recognize the platform. The rats were placed in the same quadrant of the water maze for every trial on Days 1 and 2, but on days 3 and 4, the start locations were randomized. Data from the 3 trials each day were used to calculate latency area under the curve. Inter-group comparisons were made using the Mann-Whitney test.

Lipid assays

Lipid analyses were performed with serum samples and chloroform-methanol (2:1) extracted fresh frozen liver and brain homogenates [43]. Total lipid content was measured using a Nile Red fluorescence-based assay [61–63], and fluorescence intensity (Ex 485/Em 572) was measured in a SpectraMax M5 microplate reader (Molecular Devices Corp., Sunnyvale, CA). Triglyceride levels were measured in lipid extracts using a commercial colorimetric assay kit. Ceramide immunoreactivity was measured by direct-binding ELISA [64] using 96-well Polysorp black plates (Nunc, Rochester, NY) [65]. In brief, lipids (50 μ l in methanol) were adsorbed to well bottoms for 2 h at room temperature, then blocked for 1 h with Superblock-TBS, and incubated with monoclonal anti-ceramide (2 μ g/ml) overnight at 4°C. Immunoreactivity was detected with horseradish peroxidase (HRP)-conjugated secondary antibody (1:10000) and enhanced chemiluminescence substrate (ECL) [65]. Luminescence was measured in a TopCount NXT (Packard, Meriden, CT). Positive control reactions included spotting known quantities of C2 or C6 synthetic ceramide into the wells. Immunoreactivity was normalized to sample protein content. Negative control reactions included substitutions with nonrelevant primary or secondary antibodies, and omission of primary or secondary antibody.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assays (Table 1)

We used qRT-PCR to measure mRNA expression as previously described [4,66,67]. In brief, tissues were homogenized in Qiazol reagent (Qiagen Inc., Valencia, CA), and total RNA was isolated using the EZ1 RNA universal tissue kit and the BIO Robot EZ1 (Qiagen, Inc., Valencia, CA). RNA was reverse transcribed using random oligodeoxynucleotide primers and the AMV First Strand cDNA synthesis kit. The resulting cDNA templates were used in probe-based qPCR amplification reactions with gene specific primer pairs as reported previously [67]. Primers were designed using ProbeFinder software (Roche, Indianapolis, IN), and target specificity was verified using NCBI-BLAST (Basic Local Alignment Search Tool). The amplified signals from triplicate reactions were detected and analyzed using the Mastercycler ep realplex instrument and software (Eppendorf AG, Hamburg, Germany). Relative mRNA abundance was calculated from the ng ratios of specific mRNA to β -actin mRNA measured simultaneously in duplex PCR reactions. Inter-group statistical comparisons were made using the calculated mRNA/ β -actin ratios.

Enzyme-linked immunosorbant assay (ELISA)

Tissue homogenates were prepared in NP-40 lysis buffer containing protease and phosphatase inhibitors, as previously described [52]. Protein concentration was measured using the bicinchonic acid assay. To examine signaling through the insulin and IGF-1 receptors and downstream through IRS-1 and Akt in liver tissue, we used a bead-based multiplex ELISA and measured immunoreactivity to the insulin receptor (IR), IGF-1 receptor (IGF-1R), IRS-1, Akt, and glycogen synthase kinase 3 β (GSK-3 β), and *pY pY 1162/1163-IR*, *pY pY 1135/1136-IGF-1R*, *pS312-IRS-1*, *pS473-Akt*, and *pS9-GSK3 β* . For these studies, liver tissue was homogenized in NP-40 lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet-P (NP-40)), containing protease and phosphatase inhibitors [43]. Samples containing 100 μ g protein were incubated with the beads, and captured antigens were detected with secondary antibodies generated in goat, and phycoerythrin-conjugated anti-goat antibody. Plates were read in a Bio-Plex 200 system (Bio-Rad, Hercules, CA). In addition, the samples were analyzed using direct binding ELISAs to measure myelin-associated glycoprotein-1 (MAG-1), Hu (neuronal marker), tau, and phospho-tau as previously described [68].

Statistical analysis

Data depicted in the tables represent the mean $\pm$ SEM for each group. The box-and-whisker plots depict the lower quartile (25th percentile)-bottom of box, median (horizontal bar), upper quartile (75th percentile), and range (i.e., the lowest value is represented by the bottom whisker, and the highest value is represented by the top whisker). Eight independent samples were included in each group. Inter-group comparisons were made with the Student T test (gene expression and immunoreactivity) or the Mann-Whitney-U test (rotarod and Morris Water Maze) using the GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA). Software generated significant p-values are shown in the graphs, and included in the tables.

RESULTS

Effects of ceramide on serum, liver, and brain lipids and ceramide levels (Table 2)

Age-associated increases in mean body weight, and the mean body and brain weights measured at the time of sacrifice were similar for C2Cer or C2DCer (negative control) treated rats (Fig. 1A–C). However, the mean fasting blood glucose (Fig. 1D), serum total lipid content (Nile Red assay), and serum ceramide immunore-activity were significantly higher in C2Cer-treated relative to C2DCer-treated control rats. Brain glucose levels (μ g/mg protein) were also significantly increased in the C2Cer– (33.6 ± 1.22) relative to C2DCer-treated controls (26.0 ± 1.49) ($p = 0.0009$). In contrast to serum, total lipid content in brain and liver were significantly lower in the C2Cer relative to control. In addition, in serum, brain, and liver, triglyceride concentrations were all significantly reduced in the C2Cer-treated relative to C2DCer-treated control rats. As observed in serum, ceramide levels in liver and brain were significantly higher in the C2Cer-treated rats. The proportional increases in ceramide levels detected in this model are consistent with the findings in previous studies of diet-induced obesity, chronic alcohol feeding or nitrosamine exposure [43,68–70]. Therefore, intraperitoneal treatment with bioactive C2Cer ceramide in the early postnatal period caused hyperglycemia, hyperlipidemia, and increased circulating and tissue levels of ceramides. The ceramides present in serum could potentially cross the blood-brain barrier and cause CNS injury and insulin resistance, and thereby result in further endogenous ceramide production and accumulation with attendant neurodegeneration.

Ceramide exposure impairs cognitive-motor functions (Fig. 2)

Rotarod tests performed at low speed revealed no significant differences in performance between the C2Cer-and C2DCer-treated controls. However, with increasing speed of rotation,

the C2Cer treated rats performed significantly worse than control, as manifested by their shorter latencies to fall (Fig. 2B,C). Morris Water maze testing demonstrated that C2Cer-treated rats exhibited significantly longer latencies for learning how to locate and land on the platform during the acquisition phase (Fig. 2D), but on the two subsequent days, their performance was not significantly different from control, indicating relative preservation of memory once the task had been learned. However, on the 4th and final day of testing, the C2Cer treated rats exhibited significantly longer latencies in locating the hidden platform from randomized quadrants of the maze (Fig. 2D). While the poorer performance on Day 1 might have been due to higher levels of anxiety in the C2Cer-treated rats [71], this phenomenon would not likely account for the significantly impaired performance on Day 4, or the deficits in motor function observed with increasing speed of the rotarod. In addition, the longer latencies measured in the C2Cer group were associated with to rapid swimming, but in varied (seemingly random) directions, particularly on Day 1, reflecting difficulty learning to locate and land on the platform, and Day 4, when the platform was hidden and the starting points were randomized. These results indicate that the C2Cer treatment caused significant abnormalities in spatial leaning and memory.

Histopathological effects of ceramide exposure (Fig. 3)

Livers from control (C2D injected) rats exhibited regular chord-like architecture with minimal or no inflammation or steatosis, whereas livers from C2Cer-treated rats also exhibited regular chord-like architecture, but had evidence of mild steatohepatitis characterized by the presence of multiple foci of lymphomononuclear cell inflammation, and scattered areas of microvesicular steatosis, apoptosis, and hepatocellular necrosis (Fig. 3A–D). In contrast, there were no consistent histopathological abnormalities detected in the brains, including cerebella, hippocampi, and temporal lobes of C2Cer- compared with C2D-treated rats (data not shown). C2Cer treatments resulted in significantly reduced total neutral lipid and triglyceride content, but significantly increased levels of ceramide in both liver and brain (Table 2).

Altered cellular protein expression in C2Cer-treated brains

Immunoreactivity corresponding to Hu (neurons), MAG-1 (oligodendrocyte myelin), Tau, and phospho-Tau (pS199/202-Tau) was measured in temporal lobe and cerebellar tissue by direct binding ELISA with results normalized to ribonuclear protein levels measured in the same samples. These studies demonstrated significantly reduced mean levels of Hu in the temporal lobe ($p = 0.0015$), and increased levels of phospho-Tau and Tau protein in the cerebellum of C2Cer-treated relative to controls (Table 3). In contrast, no significant inter-group differences were measured with respect to MAG-1 in either temporal lobe or cerebellum, Hu in the cerebellum, or phospho-tau and tau in the temporal lobe.

Ceramide treatment alters expression of insulin and IGF signaling genes in liver and brain

We used qRT-PCR analysis to quantify long-term effects of ceramide exposure on gene expression corresponding to insulin and IGF polypeptides and receptors, and insulin receptor substrate (IRS) molecules that transmit signals required for growth, survival, energy metabolism, and neuronal plasticity downstream of the insulin and IGF receptors (Fig. 4). Early C2Cer exposure significantly reduced insulin, IGF-2, IRS-1, and IGF-1 receptor gene expression, and increased insulin receptor and IRS-2 expression in liver. In addition, C2Cer treatments resulted in significantly reduced mRNA expression of insulin and IGF-1 receptor, and increased expression of IGF-1 in brain (Fig. 5). In contrast to liver, IRS gene expression in brain was not significantly modulated by C2Cer exposure.

Effects of ceramide exposure on insulin and IGF signaling mechanisms in liver and brain (Figs 6–8)

Multiplex bead-based ELISAs were used to measure sustained effects of C2Cer treatment on insulin and IGF signaling mechanisms in liver and brain. We measured total and phosphorylated levels of insulin receptor (pYpY1162/1163), IGF-1 receptor (pYpY1135/1136), IRS-1 (pS312), Akt (pS473), and GSK-3 β (pS9), and calculated the phospho-/total ratios to assess relative levels of phosphorylation. C2Cer treatments did not significantly alter the mean levels of total or phosphorylated insulin receptor, IGF-1 receptor, IRS-1, or Akt, but reduced the relative levels of IRS-1 and Akt phosphorylation in liver (Fig. 6). In addition, C2Cer-exposed livers had significantly reduced levels of total GSK-3 β , and increased mean levels of p^{S9} -GSK-3 β and p^{S9} -GSK-3 β /GSK-3 β relative to C2DCer-treated livers. With respect to the brain, the C2Cer exposures resulted in significantly reduced mean levels of Akt, $p^{YpY1162/1163}$ -insulin receptor/total insulin receptor, and p^{S312} -IRS-1/total IRS-1, and increased levels of insulin receptor and IRS-1 immunoreactivity in the cerebellum (Fig. 7). In contrast, in the temporal lobe, the sustained effects of C2Cer treatment on insulin/IGF signaling mechanisms were more limited in that p^{S473} -Akt/total Akt was significantly lowered, while total IRS-1 protein immunoreactivity was significantly increased (Fig. 8).

DISCUSSION

The current study represents an extension of previous work demonstrating that in various disease states of peripheral insulin resistance, including diet-induced obesity and nitrosamine exposure, the expression of several genes regulating ceramide production via *de novo* biosynthesis or sphingomyelin degradation pathways was increased in liver, and ceramide levels (immunoreactivity) were increased in liver and/or blood [2,43,51,69]. Importantly, these abnormalities were associated with brain insulin resistance and mild neurodegeneration [43, 52]. For example, in experimental diet-induced obesity with T2DM and NASH, ceramide gene expression was shown to be increased in liver, and accompanied by mild neurodegeneration with brain insulin/IGF resistance [43]. With alcoholic nitrosamine-induced steatohepatitis, pro-ceramide gene expression in liver was correlated with hepatic and brain insulin/IGF resistance and tissue injury [2,69]. Finally, *in vitro* experiments demonstrated that direct exposure to cytotoxic ceramides impairs liver and brain cell viability, mitochondrial function, and insulin/IGF signaling mechanisms [51], consistent with previous reports [10,12,13,17,¹⁸, 29,43,51,72].

The main objective of this study was to demonstrate the potential role of cytotoxic ceramides originating from the periphery as mediators of neurodegeneration. We and others have shown that in peripheral insulin resistance diseases associated with brain insulin resistance and neurodegeneration, serum and hepatic levels of ceramides, and other toxic lipids are increased [2,43,51,69]. Moreover, we demonstrated that *in vitro* cytotoxic ceramide exposure causes neurodegeneration with impaired viability, energy metabolism, and insulin/IGF signaling in neuronal cells [51]. Although generally longer chain ceramides have been detected in insulin-resistance disease models and humans [27,33,72–75], in our studies we used relatively short-chain synthetic ceramides because this approach has been validated in a number of experimental models, and the compounds are known to be cell permeable, impair signaling, and promote inflammation, mitochondrial dysfunction, and cell death [39,46,48,49,51,76], all of which are features of insulin resistance diseases. Future studies will employ longer chain synthetic ceramides, once their bio-distributions and cell permeability characteristics have been determined.

In the current study, we did not regenerate models of obesity, T2DM, or NASH, and instead focused on testing the hypothesis that peripherally administered cytotoxic ceramides can cause brain insulin resistance with impairments of cognitive and motor functions. Therefore, we

examined the degree to which limited early-life bioactive ceramide exposure leads to both hepatic and CNS insulin/IGF resistance and neurodegeneration. This study is novel because it directly examines the role of extra-CNS ceramides in relation to neurobehavioral deficits and brain insulin resistance in the absence of confounders produced by chronic high dietary fat intake, exposures to alcohol or toxins, and aging. The expectation was that ceramide exposure would impair insulin/IGF-1 signaling mechanisms in liver and brain, irrespective of peripheral blood indices of insulin resistance because the toxic lipids were deemed the culprits rather than hyperglycemia or hyperlipidemia *per se*. Correspondingly, in this study, C2Cer-induced hyperglycemia and hyperlipidemia were modest, and triglyceride levels were in fact reduced in serum, yet serum, liver, and brain ceramide levels were significantly increased. The significance of this work is that it helps establish mechanistic links among hepatic insulin/IGF resistance, lipotoxicity states, cognitive impairment, and neurodegeneration associated with deficits in brain insulin/IGF signaling. Moreover, the findings suggest that examining peripheral blood levels of ceramides may aid in identifying individuals at risk for developing cognitive impairment in the setting of obesity, irrespective of traditional biomarkers of type 2-diabetes and peripheral insulin resistance.

The over-arching hypothesis is that ceramides, which are recognized mediators of insulin resistance with demonstrated inhibitory effects on PI3K-Akt signaling [14,15], may mediate neuro-cognitive deficits, brain insulin resistance, and neurodegeneration in the context of obesity, T2DM, and NASH. In this regard, we propose that ceramides generated from the periphery (i.e., liver or perhaps adipose tissue), cross the blood-brain barrier to mediate these adverse effects on brain structure and function. The lipid-soluble nature of ceramides makes it feasible for this class of lipids to regulate and alter brain function. This phenomenon could explain how obesity and T2DM pose increased risk of cognitive impairment and neurodegeneration in humans [77–80]. In the present study, since it is now known which species of ceramides may be responsible for neurodegeneration in obesity and T2DM, and previous studies demonstrated that specific cell permeable synthetic ceramides impair insulin/IGF signaling and are cytotoxic *in vitro* [2,13–15,17,18,51], we utilized synthetic bioactive (C2Cer) and inactive (C2DCer) ceramide molecules to test our hypothesis.

Corresponding to previous findings that ceramides promote insulin resistance [2,13–15,17,18,51], we observed that following i.p. treatment of rat pups with synthetic C2Cer, as adolescents, the rats exhibited mild hyperglycemia and hyperlipidemia accompanied by increased serum ceramide levels. In addition, the livers showed evidence of on-going inflammation and injury with reduced lipid content but increased ceramide levels, and the brains exhibited normal histology, but had reduced lipid content and increased ceramide levels as well. The somewhat unexpected finding of reduced triglyceride levels in brain, liver and serum of C2-Ceramide treated versus control rats is consistent with results in another recent study in which it was suggested that ceramide treatments may mediate this effect by inhibiting adipogenesis [81].

In most cells, lipids are mainly localized in membranes and have key roles in intracellular signaling [13,17,82]. Lipid homeostasis is regulated in part by insulin signaling [13,17,82].

Degradation of sphingolipids promotes ceramide generation, which can have adverse effect on intracellular signaling, cell survival, and inflammatory mediators [13]. Correspondingly, we detected significant reductions in neuronal Hu expression in the temporal lobes of C2Cer-treated rats. This finding corresponds with the impairments in spatial learning and memory detected by Morris water maze testing. In the brain, myelin is the most abundant lipid, and myelin maintenance via oligodendroglial metabolism, is regulated by insulin and IGF signaling [83]. Although we did not detect any reductions in MAG-1 immunoreactivity, conceivably, the impairments in insulin signaling effectuated by the C2Cer treatments, and as demonstrated

in previous experiments [51], led to dysregulated lipid metabolism and increased ceramide generation, which in turn, further impaired insulin/IGF signaling mechanisms, and at least in liver, also impaired cell survival.

Although there were no overt histopathological abnormalities detected in brain, the reductions in neutral lipid and triglyceride, and increased ceramide levels may reflect the early stage of neurodegeneration. Correspondingly in previous studies, it was demonstrated that relatively early abnormalities in AD include white matter atrophy and increased ceramide content in brain [26,84]. Moreover, the impairments in cognitive and motor functions were also associated with reductions in Hu (reflecting neuronal injury or loss), and increased levels of phospho-tau and tau immunoreactivity in brains of C2Cer-treated rats. Therefore, neurobehavioral (functional), biochemical, and molecular abnormalities in brain may provide more sensitive indices of neurodegeneration, and precede many of the structural changes detected by histopathological examination. Even in humans with mild cognitive impairment, structural neurodegenerative lesions are often mild, focal, or absent [85–87]. As ceramides and other toxic sphingolipids are generated by myelin breakdown or altered biosynthesis, virtually any pathophysiological process that leads to their accumulation would also impair CNS function. Therefore, we interpret the CNS functional impairments to be consequential to combined effects of neuronal loss/degeneration precipitated by C2Cer-mediated insulin/IGF resistance, and attendant locally increased ceramide generation. At this point, it is not possible to know the relative contributions of exogenous versus endogenous ceramides mediating brain insulin resistance and neurobehavioral deficits; nonetheless, what is clear is that the neurodegeneration process can be initiated by cytotoxic ceramides generated in the periphery, i.e. outside of the CNS. While ceramide levels are increased in sera, skeletal muscle, adipose tissue, and/or liver in peripheral insulin resistance diseases [13,16,18,41,43,88,89], and in AD brains [90–92], the levels cannot be accurately quantified for comparison with our experimental model due to heterogeneity of the expressed or accumulated ceramides and other sphingolipids and the lack of standardized methods for measuring such compounds.

The molecular and biochemical studies demonstrated that the early postnatal treatment with C2Cer impaired insulin and IGF signaling mechanisms in both livers and brains of the adolescent rats. The major impact was on the expression and/or phosphorylation state of the insulin receptor, IGF-1 receptor, IRS-1 or Akt. These findings are consistent with previous reports demonstrating that ceramides impair insulin signaling through the Akt pathway [14, 15,39,93], and also with our previous findings that bioactive synthetic ceramides (C2Cer or C6Cer) impair insulin/IGF signaling through inhibition of receptor and IRS expression and function.

The insulin/IGF-1-IRS-1-Akt signaling pathway mediates cell survival, energy metabolism, neuronal plasticity, and neurotransmitter function [53]. Therefore, the observed C2Cer-mediated impairments of this pathway could account for the observed ongoing hepatocellular injury and cognitive-motor deficits. It is noteworthy that we detected increased levels of IRS-1 and/or insulin receptor protein vis-à-vis reduced relative levels of phosphorylated receptor and IRS-1. Conceivably, the increased protein levels reflect a compensatory protective response to the impairments in signaling that limited the degree and rate of cellular injury and death. The same argument could be made for the seemingly paradoxical increases in pS9-GSK-3 β in C2Cer-exposed livers.

Although the magnitudes of these effects were variable, the aggregate effects of C2Cer treatment were to reduce insulin $\pm$ increase insulin receptor gene expression, and reduce IGF-1 receptor gene expression in liver and brain, inhibit signaling downstream through IRS-1 and Akt with increasing GSK-3 β activity in liver, and constitutively impairing insulin signaling at the level of the receptor, IRS-1, or Akt in the brain. These results, together with the reduced

levels of insulin gene expression in brain, are reminiscent of the findings of both insulin resistance and insulin deficiency in brains with AD [7,8]. On the other hand, this model clearly does not replicate the abnormalities in AD, and instead seems more closely aligned with the effects of obesity and peripheral insulin resistance [43,52,68,70]. However, in future studies, it will be of interest to examine the effects of aging in relation to ceramide-mediated neurodegeneration.

In conclusion, this study demonstrates that limited *in vivo* exposure to bioactive toxic ceramides causes mild diabetes mellitus with hyperlipidemia, hepatocellular injury, deficits in cognitive and motor functions, and impairments in insulin/IGF signaling through IRS-1 and Akt. The importance of this work is that it demonstrates that peripherally generated ceramides, such as occurs in obesity, T2DM, alcoholic liver disease, and nitrosamine exposure [1,2,68,69] can mediate cognitive impairment with deficits in brain insulin/IGF signaling that promote neurodegeneration. The results support our hypothesis that in peripheral insulin resistance disease states, cognitive impairment can be mediated via a liver/peripheral-brain axis of neurodegeneration due to increased ceramide production and trafficking across the blood-brain barrier. The consequential brain insulin resistance establishes a reverberating loop of neurodegeneration whereby inhibition of signaling through insulin and IGF receptors, IRS, and Akt, perturbs energy metabolism, lipid and cholinergic homeostasis, and neuronal plasticity. The findings suggest that individuals with peripheral insulin resistance diseases who are at risk for developing cognitive impairment and neurodegeneration may be identified by examining peripheral blood and possibly cerebrospinal fluid levels of ceramides and other toxic sphingolipids, and that preventive/treatment measures could include the use of agents that reduce or block the synthesis and accumulation of such compounds.

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Fig. 1.

Effects of C2Cer on (A) growth, (B) body weight, (C) brain weight, and (D) blood glucose. Long Evans rat pups were given 7 alternate day intra-peritoneal (i.p.) injections of 2.0 mg/kg (50 μ l volume) ceramide analogs, D-erythro-N-Acetyl-Sphingosine (C2 Cer-bioactive) or D-erythro-Dihydro-N-Acetyl-Sphingosine (C2 Dihydroceramide; C2D-inactive), beginning on postnatal day 3 (P3). Body weight was measured at the time of treatment. Rats were sacrificed on P30 after an overnight fast. Blood glucose was measured with a standard glucometer. Box plots depict 25th (lower edge) and 75th (upper edge) percentiles and median (horizontal bar), and whiskers depict range. Between-group comparisons were made using Student T-tests.

Fig. 2.

C2Cer exposure impairs CNS function. Long Evans rats treated with C2Cer or C2DCer (control) were subjected to (A–C) rotarod testing on a Rotamex-5 to assess motor function, and (D) Morris water maze testing of spatial learning and memory (see Methods). For rotarod testing, rats ($n=10$ per group) were administered 10 trials at incremental speeds, and the latency to fall was automatically recorded with photocells. Data from (A) trials 1–3 (1–2 rpm), (B) 4–7 (2.5–3.5 rpm), or (C) 8–10 (4 rpm) were culled and analyzed using the Mann-Whitney test. (D) Morris Water Maze testing was performed on 4 consecutive days with 3 trials per day, beginning on P25. Day 1- rats were oriented to the maze; Day 2- rats learned to locate the hidden platform; Day 3- rats learned to locate the hidden platform from different or randomized quadrants. Data from the 3 trials each day were used to calculate area under the curve for latency. Between-group comparisons were made using the Mann-Whitney test.

Fig. 3. C2Cer treatment causes mild steatohepatitis with on-going hepatocellular injury and apoptosis. Long Evans rats were given i.p. injections of C2Cer-bioactive or C2D-inactive synthetic ceramide. Liver tissues harvested at sacrifice on P30, were fixed and paraffin embedded. Histological sections were stained with H&E. A regular chord-like hepatic architecture was observed in both (A) C2Cer- and (B) C2DCer-treated rats, but the C2Cer-exposed livers also exhibited multiple tiny foci of (C) lympho-mononuclear inflammation, (D) small clusters of hepatocytes with microvesicular steatosis, and (E) scattered foci of necrosis or apoptosis. (F) C2DCer-exposed livers were histologically intact and free of abnormalities.

Fig. 4.

C2Cer treatments impair insulin and IGF signaling gene expression in liver. Long Evans rats were given i.p. injections of C2Cer-bioactive or C2D-inactive synthetic ceramide. Liver tissues harvested on P30 was used for qRT-PCR analysis of gene expression using Duplex technology in which β -actin was co-amplified as a control gene to simultaneously assess template abundance. Box plots depict 25th (lower edge) and 75th (upper edge) percentiles and median (horizontal bar), and whiskers depict range. Between-group comparisons were made using the Student T-test.

Fig. 5.

C2Cer treatments impair insulin and IGF signaling gene expression in brain. Long Evans rats were given i.p. injections of C2Cer-bioactive or C2D-inactive synthetic ceramide. Cerebellar tissues harvested on P30 was used for qRT-PCR analysis of gene expression using Duplex technology in which β -actin was co-amplified as a control gene to simultaneously assess template abundance. Box plots depict 25th (lower edge) and 75th (upper edge) percentiles and median (horizontal bar), and whiskers depict range. Between-group comparisons were made using the Student T-test.

Fig. 6. C2Cer treatments impair insulin and IGF signaling mechanisms in liver. Long Evans rats were given i.p. injections of C2Cer-bioactive or C2D-inactive synthetic ceramide. Liver tissue homogenates were used to measure (A) insulin receptor, (B) IGF-1 receptor, (C) IRS-1, (D) Akt, (E) GSK-3 β , (F) $pYpY1162/1163$ -Insulin receptor (IN-R), (G) $pYpY1135/1136$ -IGF-1 receptor, (H) $pS312$ -IRS-1, (I) $pS473$ -Akt, (J) $pS9$ -GSK-3 β immunoreactivity by multiplex bead-based ELISA. The phosphorylated/total (K) insulin receptor, (L) IGF-1 receptor, (M) IRS-1, (N) Akt, and (O) GSK-3 β ratios were calculated to assess relative levels of protein phosphorylation. Box plots depict 25th (lower edge) and 75th (upper edge) percentiles and median (horizontal bar), and whiskers depict range. Between-group comparisons were made using the Student T-test.

Fig. 7.

C2Cer treatments impair insulin and IGF signaling mechanisms in the cerebellum. Long Evans rats were given i.p. injections of C2Cer-bioactive or C2D-inactive synthetic ceramide.

Cerebellar tissue homogenates were used to measure (A) insulin receptor, (B) IGF-1 receptor, (C) IRS-1, (D) Akt, (E) GSK-3 β , (F) pYpY 1162/1163 -Insulin receptor (IN-R), (G) pYpY 1135/1136-IGF-1 receptor, (H) pS312-IRS-1, (I) pS473-Akt, (J) pS9-GSK-3 β immunoreactivity by multiplex bead-based ELISA. The phosphorylated/total (K) insulin receptor, (L) IGF-1 receptor, (M) IRS-1, (N) Akt, and (O) GSK-3 β ratios were calculated to assess relative levels of protein phosphorylation. Box plots depict 25th (lower edge) and 75th

(upper edge) percentiles and median (horizontal bar), and whiskers depict range. Between-group comparisons were made using the Student T-test.

Fig. 8. C2Cer treatments impair insulin and IGF signaling mechanisms in the temporal lobe. Long Evans rats were given i.p. injections of C2Cer-bioactive or C2D-inactive synthetic ceramide. Temporal lobe tissue homogenates were used to measure (A) insulin receptor, (B) IGF-1 receptor, (C) IRS-1, (D) Akt, (E) GSK-3 β , (F) pYpY^{S1162/1163}-Insulin receptor (IN-R), (G) pYpY^{S1135/1136}-IGF-1 receptor, (H) pS³¹²-IRS-1, (I) pS⁴⁷³-Akt, (J) pS⁹-GSK-3 β immunoreactivity by multiplex bead-based ELISA. The phosphorylated/total (K) insulin receptor, (L) IGF-1 receptor, (M) IRS-1, (N) Akt, and (O) GSK-3 β ratios were calculated to assess relative levels of protein phosphorylation. Box plots depict 25th (lower edge) and 75th

(upper edge) percentiles and median (horizontal bar), and whiskers depict range. Between-group comparisons were made using the Student T-test.

Table 1

Primer pair sequences and probes used for quantitative PCR*

Primer	Sequence 5' to 3'	Position	UPL probe #
rINS-F	CCGGTGACCTTCAGACCTT	221	10
rINS-R	TGCTGGTGCAGCACTGAT	278	
rIGF1-F	CGGTTGCACTAAATTCCTCTCT	829	66
rIGF1-R	CATCAACCACCTATAAACAGTTGC	880	
rIGF2-F	GTCACCCATGTCACCAAGG	2231	55
rIGF2-R	AGGTTCTATTTTCAGTTTTCATGTTCT	2380	
rINS Rec-F	CTCTGCCTGTCTGAGATCCAC	1725	69
rINS Rec-R	GCAATGTCGTTCTCTCCTG	1779	
rIGF1Rec-F	CTCTGCCTTTTCTCTCTCTG	4483	55
rIGF1Rec-R	GAGGAGAGCCTGGAAGTGG	4529	
rIGF2Rec-F	AACAAAACCGCAGGTCAAGA	1359	55
rIGF2Rec-R	TGCAGTCCACCTCGATTGT	1401	
rIRS1 -F	TCACGATTACAACCAGGAC	4386	69
rIRS1-R	AGGGATGCATCGTACCATCT	4426	
rIRS2-F	AGTTCAGGTCGCCTCTGC	1813	55
rIRS2-R	TCTGGGTAAGGGTTGTAGGC	1861	
rIRS4 -F	TCACTCAAAGCAAGCAGCA	2197	78
rIRS4-R	ACTTCCCTTTGCCACCAGT	2274	

* Abbreviations: r = rat; IGF-1 = insulin-like growth factor, type 1; INS = insulin; Rec = receptor; IRS = insulin receptor substrate; F = forward primer; R = reverse primer; UPL = universal probe library number.

Table 2

Lipid profiles associated with ceramide exposure. Effects of C2Cer on total lipid, triglyceride, and ceramide levels in serum, cerebellar, temporal lobe, and liver tissue. Long Evans rat pups were treated with ceramide analogs, D-erythro-N-Acetyl-Sphingosine (C2 Cer-bioactive) or D-erythro-Dihydro-N-Acetyl-Sphingosine (C2 Dihydroceramide; C2D-inactive) by i.p. injection (see Methods). Lipids were measured using the Nile Red assay. Triglyceride content (μg) was measured using a standard assay kit. Ceramide immunoreactivity was measured by ELISA (FLU=fluorescence light units). Measurements were made using serum or lipid extracts of the tissues. Results were normalized to volume or protein content in the tissue samples. Data represent mean $\pm$ SEM of results, and significant p-values generated by T-test analysis are indicated

Nile Red (FLU)	C2Cer	C2DCer	p-value
Serum	414.1 $\pm$ 24.58	334.1 $\pm$ 27.86	0.0182
Cerebellum	11.01 $\pm$ 0.59	12.68 $\pm$ 0.71	0.038
Temporal Lobe	15.46 $\pm$ 1.36	20.22 $\pm$ 1.54	0.012
Liver	13.38 $\pm$ 1.21	22.45 $\pm$ 1.23	< 0.0001
Triglyceride	C2Cer	C2DCer	p-value
Serum	0.2529 $\pm$ 0.0251	0.3246 $\pm$ 0.0269	0.032
Cerebellum	0.03719 $\pm$ 0.0015	0.0447 $\pm$ 0.0024	0.007
Temporal Lobe	0.03194 $\pm$ 0.00116	0.03114 $\pm$ 0.0015	
Liver	0.2367 $\pm$ 0.0152	0.3281 $\pm$ 0.0221	0.001
Ceramide (FLU)	C2Cer	C2DCer	p-value
Serum	299129 $\pm$ 1.77E+04	194714 $\pm$ 1.37E+04	< 0.0001
Cerebellum	324796 $\pm$ 1.81E+04	285862 $\pm$ 1.63E+04	0.018
Temporal Lobe	182864 $\pm$ 1.33E+04	147743 $\pm$ 1.28E+04	0.035
Liver	1524000 $\pm$ 8.58E+04	1144000 $\pm$ 4.66E+04	0.0004

Table 3
Effects of C2Cer Treatment on Brain Neuronal and Oligodendrocyte Protein Expression

Long Evans rat pups were treated with ceramide analogs, D-erythro-N-Acetyl-Sphingosine (C2Cer-bioactive) or D-erythro-Dihydro-N-Acetyl-Sphingosine (C2 Dihydroceramide; C2DCer-inactive) by i.p. injection (see Methods). Protein homogenates prepared in NP-40 lysis buffer were used to measure immunoreactivity in direct binding ELISAs with results normalized to ribonuclear protein level in each well. Results represent the mean $\pm$ S.E.M. of the calculated ratios of immunoreactivity. Inter-group statistical comparisons were made using Student T-tests and significant p-values are indicated

Protein	Temporal lobe			Cerebellum		
	C2Cer	C2DCer	p-value	C2Cer	C2DCer	p-value
Hu	12.53 $\pm$ 1.01	17.19 $\pm$ 0.95	0.0015	20.68 $\pm$ 0.43	19.98 $\pm$ 0.71	
MAG-1	23.20 $\pm$ 0.98	22.07 $\pm$ 1.33		15.46 $\pm$ 0.70	14.15 $\pm$ 0.57	
pTau	25.38 $\pm$ 1.25	27.76 $\pm$ 1.67		26.14 $\pm$ 1.59	19.86 $\pm$ 0.74	0.0005
Tau	410.10 $\pm$ 16.60	411.00 $\pm$ 15.89		342.90 $\pm$ 12.73	306.50 $\pm$ 9.90	0.02