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Received Date : 28-Oct-2016

Revised Date : 10-Jan-2017

Accepted Date : 16-Jan-2017

Article type : Original Article

Differential regulation of sphingolipid metabolism in plasma, hippocampus and cerebral cortex of mice administered sphingolipid modulating agents.

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Running Title: Sphingolipid modulation in plasma and brain

Keywords: Ceramide, Lipidomics, Brain, Mass Spectrometry, Neurodegeneration

Abbreviations: CTX, cerebral cortex; HPF, hippocampus; CTRL, control diet; SFA, saturated fat enriched diet; MYR, myriocin; AMI, amitriptyline; FTY, FTY 720; SPT, serine-palmitoyltransferase; aSMase, acid sphingomyelinase; AD, Alzheimer's disease; BBB, blood-brain barrier; Cer(d18:1), ceramides; Cer(d18:0), dihydroceramides; SM, sphingomyelin; HexCer, monohexosylceramides; Hex2Cer, dihexosylceramides; Hex3Cer, trihexosylceramides; GM3, GM3 ganglioside; S1P, sphingosine-1-phosphate.

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Accumulation of ceramide is implicated in mediating the cellular responses to stress and aberrant sphingolipid metabolism is frequently associated with metabolic and neurodegenerative diseases. It is often assumed that (1) peripheral disturbances in sphingolipid concentrations are reflective of processes occurring in the brain, or (2) circulating sphingolipids directly influence cerebral sphingolipid abundance. In order to address these assumptions, this study explores, in a physiological system, the metabolic pathways regulating sphingolipid metabolism in the brain and plasma of mice. Male C57Bl/6 were maintained on a low fat (CTRL) or saturated fat enriched (SFA) diet with, or without the provision of sphingolipid modulating agents. Following six months of feeding, the abundance of seven sphingolipid classes was assessed by LC-ESI-MS/MS in the hippocampus (HPF), cerebral cortex (CTX) and plasma. Long-term consumption of the SFA diet increased ceramide and dihydroceramide in the plasma. Inhibiting de novo synthesis ameliorated this effect, while inhibition of acidic sphingomyelinase, or the sphingosine-1-phosphate receptor agonist did not. SFA feeding did not influence sphingolipid levels in either the HPF or CTX. De novo synthesis inhibition reduced ceramide in the CTX, whilst treatment with a sphingosine-1-phosphate receptor agonist reduced ceramides in the HPF. Analysis of the individual ceramide species revealed the effects were chain-length dependent. Both positive and negative correlations were observed between plasma and HPF/CTX ceramide species. The findings in this study show that HPF and CTX sphingolipid

concentration are influenced by distinct pathways, independent of peripheral sphingolipid concentration.

Introduction

Sphingolipids are highly enriched in the central nervous system, contributing to the structural integrity and fluidity of cellular membranes and myelin sheaths. Accumulating evidence suggests that sphingolipid metabolism is perturbed early in the development of many neurodegenerative diseases, including Alzheimer's disease (AD) (Cutler *et al.* 2004), Multiple Sclerosis (Vidaurre *et al.* 2014) and Parkinson's disease (Mielke *et al.* 2013, Xing *et al.* 2016). Several post-mortem studies have found that elevated levels of ceramides are found in the brain of AD patients (Cutler et al. 2004, Han *et al.* 2002, Filippov *et al.* 2012). The susceptibility of neurons to ceramide-induced pathology has been extensively reviewed (Jana *et al.* 2009, Mencarelli & Martinez-Martinez 2013). Interestingly, increases in plasma ceramides have been shown to predict cognitive decline and hippocampal volume loss in patients with mild cognitive impairment (Mielke *et al.* 2010b). Furthermore, elevated circulating ceramides were associated with a worsening of white matter microstructure in cognitively normal patients (Gonzalez *et al.* 2016). These studies highlight the importance of sphingolipid metabolism for central nervous system function and the potential for peripheral ceramides to influence the brain. Despite the accumulating evidence suggesting a pivotal role of sphingolipid deregulation in pathogenesis of many brain disorders, few studies have attempted pharmacological modulation of sphingolipid abundance in the brain, nor attempted to examine the potential of circulating ceramides to influence cerebral ceramide concentrations.

Ceramides have been implicated as a lipid mediator of cellular responses, including redox homeostasis, inflammation and indeed apoptosis (Hannun 1996). Forming the central metabolite in sphingolipid metabolic network, ceramides exist as both ubiquitous modulators of membrane dynamics and as a second messenger (van Blitterswijk *et al.* 2003). Therefore, it is important to consider the multiple pathways that regulate ceramide metabolism. In cellular systems, two principle pathways have been extensively studied that are involved in ceramide accumulation – de novo synthesis, a non-reversible pathway catalysed by the rate limiting enzyme serine-palmitoyltransferase (SPT); and sphingomyelin hydrolysis by sphingomyelinases. However, in physiological systems, there is potential for trafficking of circulating ceramides to organs through uptake of lipoprotein-associated ceramides and lipid soluble exchange during lipoprotein-cell interactions (Boyanovsky *et al.* 2003, Boon *et al.* 2013). There have been several suggestions that plasma ceramides are associated with cerebrospinal fluid (Mielke et al. 2010b, Mielke *et al.* 2012,

Mielke *et al.* 2011). Given that circulating ceramides appear early in the development of many neurodegenerative diseases, their contribution to deregulation of sphingolipid metabolism in the brain should be clarified.

A family of ceramide synthases (CerS), involved during de novo synthesis, are responsible for the differences in acyl-chain lengths across tissues (Levy & Futerman 2010). Six CerS catalyse the N-acylation of acyl-CoAs to the (dihydro)sphingosine backbone, each utilising a restricted set of acyl-CoAs. In addition to their involvement in de novo synthesis, CerS also have central roles in the sphingolipid recycling or "salvage pathway" (Kitatani *et al.* 2008). There have been reported differences in the acyl-chain composition of sphingolipids between blood, cerebrospinal fluid and brain tissue (Haughey *et al.* 2004, Mielke *et al.* 2015) and its conceivable that circulating ceramides may exert their biological effects after delivery and deacylation/reacylation. The involvement and role of ceramide acyl chain length and ceramide synthases in neurodegeneration has recently been reviewed (Ben-David & Futerman 2010).An increase in the number of studies examining the role of sphingolipids in pathologies has occurred due to the discovery of pharmacological agents that modulate different pathways of sphingolipid metabolism. These sphingolipid modulating agents are in use in animal and human research. Myriocin (MYR), an inhibitor of SPT, has been shown to decrease atherosclerosis in apo-E deficient mice (Hojjati *et al.* 2005); ameliorate glucocorticoid-, saturated fat- and obesity induced insulin resistance (Holland *et al.* 2007); and to reduce non-alcoholic fatty liver disease in high-fat fed rats (Kurek *et al.* 2014). Inhibitors of sphingomyelinases typically fall under two categories, acidic sphingomyelinases, or neutral sphingomyelinase inhibitors. Amitriptyline (AMI), an inhibitor of acid sphingomyelinase (aSMase), has been used in the study of cystic fibrosis (Teichgraber *et al.* 2008, Becker *et al.* 2010), renal injury during high-fat diets (Boini *et al.* 2010), and studied for the role of sphingomyelinase inhibition in anti-depressant drugs (Gulbins *et al.* 2013).

Although much research has focused on inhibiting ceramide accumulation, the phosphorylated sphingosine base, sphingosine-1-phosphate (S1P), opposes many of the cellular functions of ceramide. While no pharmacological agent is routinely in use to increase ceramide degradation, there are S1P mimetics in use in clinical trials. FTY720 (FTY) is the first oral drug approved for treatment of the relapsing-remitting form of multiple sclerosis and works through activation of sphingosine-1-phosphate receptors (Brinkmann *et al.* 2002). There is currently debate as to the physiological mechanism through which FTY abates multiple sclerosis and other neurodegenerative disorders. Whether the mechanism is principally through a peripheral immunomodulatory response, or through direct actions on neurons and cerebrovascular protective effects (van Doorn *et al.* 2012).

Furthermore, the ceramide modulating effects of FTY have been noted in the periphery (Bruce *et al.* 2013) but require clarification, especially in the brain.

In this study, concentrations of multiple sphingolipid classes and species are measured in plasma, HPF and CTX of an animal model recognised for peripheral disturbances of sphingolipid metabolism and which shows early pathological symptoms of neurovascular inflammation (Takechi *et al.* 2014). To consider the putative role of the different pathways that regulate sphingolipids, a suite of sphingolipid modulating agents were utilised, shown to attenuate ceramide accumulation in peripheral organs through alternate pathways. In addition, the associations between plasma ceramides and HPF and CTX ceramides are reported.

Methods

Animals

All experimental procedures were approved by the Curtin University Animal Ethics Committee and completed in accordance with National Health and Medical Research Council (NHMRC) guidelines. Male C57BL/6 mice where obtained from the Animal Resource Centre (ARC; Murdoch, Western Australia). They were housed in an accredited animal holding facility with 12 hour light/dark cycles, at ambient temperature of 22 degrees Celsius. Mice had ad libitum access to water and their respective diets. At 8 weeks of age, mice were randomly assigned to one of five groups (n=10 per group), receiving either a control diet (CTRL, AIN-93M), a modified AIN-93M chow containing 40% energy from cocoa butter (SFA), SFA diet with MYR, SFA diet with AMI or SFA diet with FTY. Myriocin was incorporated into chow at 1.5 mg/kg (w/w) to deliver a daily dose of approximately 0.3 mg/kg body weight. Amitriptyline and FTY were supplied through drinking water, at concentrations of 125 mg/L and 3.75 mg/L, respectively. Fresh drinking water solutions were prepared daily.

Sample isolation and preparation

Mice were maintained on their respective diets and treatments for six months. Mice were administered an intraperitoneal dose of pentobarbital and following complete anaesthesia, blood was collected through cardiac puncture into EDTA containing tubes. Plasma was separated through centrifugation and frozen at -80 degrees Celsius for further analysis. Following exsanguination, brains were rapidly excised, washed in ice-cold PBS and left hemispheres snap frozen in liquid nitrogen. Using a commercial brain block, the left frozen hemispheres were sectioned into 1 mm

coronal slices. Under a stereotaxic microscope, sections of the S2 cerebral cortex and hippocampus were isolated and weights recorded. Isolated regions were diluted in 10 volumes of ice cold phosphate-buffered saline (10 mM phosphate, 137 mM NaCl, 2.7 mM KCl; pH 7.4), homogenized and frozen for further analysis.

Extraction of lipids

The extraction of lipids was completed by a signle phase chloroform:methanol extraction as described previously (Weir *et al.* 2013). In brief, 10 uL of plasma or 10 uL of brain homogenate (containing approximately 20 ug of protein) was transferred to an eppendorf tube with 10 uL of internal standard mixture (Weir et al. 2013). Chloroform/methanol (2:1; 20 volumes) was added to each sample, followed by rotary mixing (10 minutes), sonication (30 minutes) and standing (20 minutes) at room temperature. Samples were centrifuged (16,000 x g, 10 minutes) and the supernatant collected and dried under nitrogen gas at 40°C. Samples were reconstituted with 50 uL of water saturated butanol and sonicated (10 minutes), followed by 50 uL methanol (with 10 mM ammonium formate). Extracts were centrifuged (3350 x g, 5 minutes) and the supernatant transferred to 0.2 mL glass vials with Teflon caps ready for analysis.

Mass spectrometric analysis of lipids

Analysis of lipids was conducted using liquid chromatography electrospray ionisation-tandem mass spectrometry on an Agilent 1200 UHPLC coupled to an AB Sciex Q/TRAP 4000 mass spectrometer with a turbo-ionspray source as extensively described previously (Weir et al. 2013). Briefly, detection of lipids was performed with scheduled multiple reaction monitoring (MRM) in positive ion mode using Analyst 1.5 (AB Sciex). The concentration of individual lipid species was determined by relating the integrated peaks to the appropriate internal standards in MultiQuant 2.1 (AB Sciex). Samples were randomised and quality control samples were analysed each 20 samples to assess assay performance. In total, 55 lipid species in 7 lipid classes and subclasses where detected and quantitated from each plasma, cerebral cortex and hippocampal sample.

Data analysis

Individual lipid species concentrations were expressed as picomoles per mg wet weight for brain samples and picomoles per microliter for plasma samples. Total lipid concentration of each class was calculated by summing the individual species in that class. All lipid species detected within a bio-compartment (hippocampus, cerebral cortex or plasma) were used for calculating class sums.

Prior to statistical comparisons, all data was tested and confirmed for normality using Shapiro-Wilk test. Univariate comparisons were conducted comparing lipids between the high-fat diet fed mice

against lipids observed in the control diet fed group and those receiving sphingolipid modulating agents. For these comparisons, independent samples t-tests were used. Correlations were assessed by Pearson's correlation.

Data analysis was performed using R version 3.3.1 (R Core Team 2015).

Results

Sphingolipid profiles were determined in the plasma, hippocampus (HPF) and cerebral cortex (CTX) of mice following six months feeding with their respective diet/drug. Sphingolipids were classified as one of seven classes and subclasses: ceramides (Cer(d18:1)), dihydroceramide (Cer(d18:0)), sphingomyelin (SM), monohexosylceramide (HexCer), dihexosylceramide (Hex2Cer), trihexosylceramide (Hex3Cer) and G_{M3} ganglioside (GM3). Table 1 details the estimated molar abundance of the sphingolipid classes in plasma.

Mice maintained on the SFA diet for six months developed an altered plasma sphingolipid composition in comparison to those on the CTRL diet. Mice on the SFA diet showed a 38.3 % increase in total Cer(d18:1) in plasma compared to CTRL animals (Table 1; $p<0.001$). Cer(d18:0), the de novo synthesis pathway precursor to Cer(d18:1), increased 326 % in plasma of mice fed the SFA diet ($p<0.001$). The plasma abundance of SM, HexCer, Hex2Cer, Hex3Cer and GM3 did not change in response to the SFA diet.

Treatment of mice with MYR, an inhibitor of sphingolipid de novo synthesis, produced a substantially altered plasma sphingolipid profile. Myriocin reduced plasma Cer(d18:1) by 42.6 % compared to mice on the SFA diet ($p<0.001$). Similarly, Cer(d18:0) was reduced by 40.4 % compared to SFA diet group ($p=0.012$). Myriocin treatment reduced plasma SM compared to the CTRL group ($p=0.024$), but did not reach statistical significance compared with SFA. Complex glycosphingolipids were also altered by treatment with MYR. Plasma HexCer was reduced compared to both the SFA and CTRL groups ($p<0.001$), while plasma Hex2Cer ($p<0.05$) and GM3 ($p<0.001$) were increased. No change in plasma Hex3Cer was observed with treatment of MYR.

Amitriptyline and FTY did not alter the plasma sphingolipid composition in comparison to SFA diet alone. Similar to the SFA only group, plasma Cer(d18:1) and Cer(d18:0) were elevated over CTRL animals (all comparisons, $p<0.001$). Plasma SM, HexCer, Hex2Cer, Hex3Cer, GM3 were not significantly altered in the AMI and FTY groups.

The HPF and CTX sphingolipid profile was characterized and differed substantially from that observed in plasma (Table 1). Comparison of sphingolipid class abundance between the HPF and CTX is shown in Figure 1. Based on the abundance per milligram of tissue, the HPF is more enriched in

sphingolipids than the CTX. Key differences between the two regions principally lie in the abundance of Cer(d18:1) ($p<0.001$), Cer(d18:0) ($p=0.002$), SM ($p<0.001$) and GM3 ($p<0.001$).

Compared to CTRL fed mice, the SFA diet did not significantly alter the abundance of any sphingolipid class in either the HPF or CTX. Mice treated with MYR showed a decrease in the abundance of Cer(d18:1) within the CTX compared to CTRL animals ($p=0.016$), although this did not reach statistical significance compared to the SFA only group ($p=0.082$). The abundance of the other sphingolipid classes were not altered by MYR in either the HPF or CTX.

Mice treated with AMI had similar levels of Cer(d18:1) in both the CTX and HPF. However, they showed increased concentrations of Hex2Cer in the HPF compared to CTRL animals ($p=0.05$). The abundance of other sphingolipid classes were not altered by AMI in both regions of the brain.

Mice consuming the SFA diet and treated with FTY showed a decreased HPF abundance of Cer(d18:1) compared to both CTRL ($p=0.004$) and SFA alone ($p=0.05$). This effect was only observed in the HPF, as the CTX abundance of Cer(d18:1) was not different compared to CTRL or SFA. The abundance of other sphingolipid classes were not significantly altered by FTY in both regions of the brain.

The accumulation of different Cer(d18:1) species has been implicated in inducing different cellular functions, therefore the abundance of individual species is analysed in the plasma, HPF and CTX. The changes in plasma Cer(d18:1) were species specific (Figure 2). Consumption of the saturated fat enriched diet caused substantial rearrangement of the plasma ceramide profile (Figure 2). Ceramide species d18:1/16:0 (↑121 %, $p<0.001$), d18:1/20:0 (↑185 %, $p<0.001$), d18:1/22:0 (↑216 %, $p<0.001$), d18:1/22:1 (↑86 %, $p=0.001$), d18:1/24:0 (↑104 %, $p<0.001$) and d18:1/26:1 (↑49 %, $p=0.002$) all increased following consumption of the SFA diet.

Treatment with MYR ameliorated much of the effects of the SFA diet on the plasma Cer(d18:1) profile, lowered elevated species to near or below CTRL levels. Treatment with AMI or FTY did not cause a significant change in the plasma ceramide profile in comparison to SFA diet alone.

The HPF (Figure 3) and CTX (Figure 4) showed a substantially different ceramide profile from that observed in plasma. In CTRL mice, the ceramide isoform d18:1/18:0 accounted for 88 % of HPF ceramides and 82 % of ceramide abundance in CTX. Intermediate abundance species included d18:1/24:1 (5.3 %, 9.9 %; HPF, CTX), d18:1/16:0 (2.3 %, 2.2 %) and d18:1/20:0 (1.7 %, 1.9 %). The other isoforms constituted minor species and each make up less than 1 % of total ceramides.

Mice fed the SFA diet did not exhibit an altered abundance of any ceramide species compared to CTRL animals, in both the HPF and CTX. Myriocin administration caused the abundance of several

ceramide species to decrease in the CTX (Figure 4). Amitriptyline treatment reduced abundance of several ceramide species in both the HPF and CTX. Whereas, mice in the FTY group had a reduced total Cer(d18:1) abundance in the HPF and this corresponded to the reduction in several ceramide isoforms.

In order to investigate the putative associations between circulating plasma ceramides and cerebral ceramide species, a correlation matrix between plasma and the two regions of the brain was generated (Fig 5). Between plasma and the hippocampus, there were numerous significant negative correlations, particularly involving plasma species d18:1/16:0, d18:1/20:0, d18:1/22:0, d18:1/22:1, and d18:1/24:0. The corresponding hippocampus species were d18:1/16:0, d18:1/18:0, d18:1/20:0, d18:1/22:0, d18:1/22:1, d18:1/24:0 and d18:1/24:1. In contrast, there was a trend to more positive correlations with the CTX. However, only one plasma specie, d18:1/24:1, was significantly correlated with any CTX species.

Discussion

In this study, a lipidomic approach was used to elucidate the metabolic pathways regulating sphingolipid concentrations in the brain and plasma of mice. Using sphingolipid modulating agents combined with an SFA enriched diet, changes in the abundance of sphingolipid classes were assessed in plasma, the HPF and CTX of each animal. Long-term consumption of the SFA diet increased Cer(d18:1) and Cer(d18:0) in plasma. Inhibiting de novo synthesis reversed these changes, while inhibiting aSMase or activating S1P receptors had no effect. In contrast to plasma, the SFA diet alone had no effect on the abundance of sphingolipids in the HPF and CTX. The data supports the hypothesis that the HPF and CTX respond differently to the sphingolipid modulating agents. De novo synthesis inhibition reduced Cer(d18:1) in the cortex, while FTY reduced Cer(d18:1) in the HPF. Analysis of the individual Cer(d18:1) species revealed that changes are chain length dependant. In addition, aSMase inhibition reduced the abundance of several ceramide isoforms in both the HPF and CTX. Lastly, the correlation between circulating and cerebral Cer(d18:1) showed both positive and negative associations depending on the species and cerebral location. This study highlights the associations, in a physiological model, between regional responses in the brain and plasma to sphingolipid modulating agents.

Circulating sphingolipids have been shown as predictive markers for risk and progression of neurodegenerative disorders. Mielke et al. (Mielke *et al.* 2010a) reported that elevated SM and

Cer(d18:1) are early predictors of memory impairment in a longitudinal study. Another study revealed that higher plasma Cer(d18:1) were associated with poorer cerebral white matter microstructure (Gonzalez et al. 2016). These studies suggest that plasma sphingolipids may have a biological role in the pathogenesis in neurodegenerative disorders. It has been speculated that peripheral sphingolipids may (1) be reflective of changes in lipid concentrations that occurs in the brain; (2) directly influence the functioning of the central nervous system, through uptake; or (3) indirectly, by altering risks factors such as peripheral insulin resistance or vascular dysfunction. However, to date, no studies have reconciled these alternate hypotheses. To address this, three sphingolipid modulating agents are used in an SFA enriched diet mouse model and the plasma, HPF and CTX sphingolipidomes are compared.

Herein, the results provide confirmatory evidence that perturbations in plasma sphingolipid concentrations with SFA feeding is principally determined through de novo synthesis. Inhibiting de novo synthesis ameliorated the elevated Cer(d18:1) and Cer(d18:0), while inhibiting aSMase and activation of S1P receptors had no effect on plasma sphingolipids. This finding support the results of previous studies (Holland et al. 2007, Hojjati et al. 2005), however contrast with the suggestion of Boini *et al.* that aSMase is a major pathway leading to Cer(d18:1) accumulation in the plasma of high-fat fed mice (Boini et al. 2010). Changes in complex sphingolipids were not observed with the SFA diet, but were with long-term MYR treatment. Reduced de novo synthesis led to reduced SM and HexCer, both of which have been implicated in neurodegenerative disorders, atherosclerosis and endothelial dysfunction (Glaros *et al.* 2008).

In contrast to plasma, there was no observed change in the total abundance of sphingolipid classes in either region of the brain, in response to SFA feeding. The finding is in contrast to Borg et al. who reported hypothalamic accumulation of Cer(d18:1), Cer(d18:0) and Hex2Cer in mice chronically fed a 60 % fat diet (Borg *et al.* 2012). The hypothalamus is a region of the brain which has 'lipid sensing' functions and previously shown to accumulate long-chain acyl-CoA in response to short term overfeeding (Pocai *et al.* 2006). Differences in the regional abundance and response of lipid supply likely account for the differences in results reported. While there were no changes in response to the SFA enriched diet, the response to the sphingolipid modulating agents revealed region specific changes in the abundance of several sphingolipid classes. In mice chronically administered FTY, HPF ceramides were decreased, while CTX ceramides were unaffected. FTY has been shown to have bioactive functions in the brain, whereby it concentrates and exceeds blood concentrations several fold (Foster *et al.* 2007). FTY has been shown to inhibit ceramide synthases (Berdyshev *et al.* 2009), to inhibit acidic sphingomyelinase (Dawson & Qin 2011) and recently shown to reduce muscle ceramide concentration in response to high-fat feeding (Bruce et al. 2013). Within the CTX, inhibition

of de novo synthesis with MYR reduced Cer(d18:1) concentration. Few studies have assessed the effects of MYR to influence cerebral sphingolipids. However, Saito et al. reported reductions in cerebral ceramides with intracerebroventricular injection of MYR in a mouse model of ethanol-induced apoptotic neurodegeneration, but did not explore the regions influenced (Saito *et al.* 2010). Inhibition of acidic sphingomyelinase, with AMI and fluoxetine, has been shown to dose dependently reduce ceramides in the hippocampus of ordinary mice. However, therapeutic treatment with fluoxetine in an induced stroke mouse model showed no effect on CTX ceramides. Similarly, in this study with SFA fed mice, there was no observed change in total Cer(d18:1) in either brain region in response to AMI treatment.

It has been proposed that some of the biological effects of ceramides might be acyl-chain dependant (Grosch *et al.* 2012, Park *et al.* 2014). Ceramide acyl chain length is determined by a set of six ceramide synthases that are specific for varying acyl-CoA chain length. The loss of a single ceramide synthase, has been shown to lead to alterations in the abundance of sphingolipids in the brain, myelin deficiency and neurodegeneration (Zhao *et al.* 2011, Imgrund *et al.* 2009). Given there is tight regulation on ceramide acyl chain length and recycling (Schiffmann *et al.* 2013), changes in individual ceramide species was assessed in plasma, hippocampus and cortex. The data presented suggests there is very tight regulation in maintaining the abundance of Cer(d18:1/18:0) in plasma. Interestingly, among the plasma ceramide species measured in a longitudinal study of elderly women, Cer(d18:1/18:0) was the poorest predictor of dementia and AD risk (Mielke et al. 2012). Acyl-chain specific changes were also observed in both regions of the brain. Cer(d18:1/16:0) and Cer(d18:1/26:0) were unaffected by the SFA diet or the sphingolipid modulated agents, in both regions of the brain. As Cer(d18:1/18:0) was the predominant isoform, a reduction in this specie likely accounts for the reduction in total ceramides. The ceramide species Cer(d18:1/18:0), Cer(d18:1/20:0), Cer(d18:1/22:0), Cer(d18:1/22:1), Cer(d18:1/24:0), Cer(d18:1/24:1) and Cer(d18:1/26:1) were reduced in at least two different groups receiving sphingolipid modulating agents, suggesting they reflect a more dynamic ceramide metabolic pool. Interestingly, while AMI treatment did not reduce total Cer(d18:1), it did reduce a number of the very long-chain ceramides in the CTX. Further, AMI reduced Cer(d18:1/24:0) in both the HPF and CTX. The latter may be significant, because cerebral levels of Cer(d18:1/24:0) is frequently found to be elevated in AD and other neurodegenerative disorders (for review see (Ben-David & Futerman 2010)).

Presently, the physiological function of circulating ceramides is unclear, despite their purported central function for development and progression of neurodegenerative diseases. The hepatic ceramide-brain axis has been proposed to link several metabolic stresses to neurodegeneration (Lyn-Cook *et al.* 2009). De la Monte et al. suggested that short-chain ceramides may cross the blood-brain

barrier and showed that intraperitoneal injection of a short-chain ceramide (Cer(d18:1/2:0) increased the concentration of ceramides in the brains of Evans Long rat pups (de la Monte *et al.* 2010). Herein, we assessed associations between circulating and brain Cer(d18:1). Contrary to the hypothesis indicated, the strongest associations were indicated with multiple negative correlations between plasma and HPF ceramide species. Although circulating Cer(d18:1) do not directly correlate with brain Cer(d18:1), it does not mean they may not be good biomarkers for neurodegenerative diseases, as suggested by Miekle and Lyketsos (Mielke & Lyketsos 2010). Nonetheless, elevated circulating Cer(d18:1) may influence the brain or cerebrovasculature without overtly raising tissue levels of ceramides. A study by Boon et al. (2013) demonstrated that circulating Cer(d18:1) was associated with biological effects in organs without elevating tissue levels per se. Through infusion of low-density lipoprotein enriched with Cer(d18:1), Boon et al. were able to singularly isolate the effects of elevated circulating Cer(d18:1) on inducing skeletal muscle insulin resistance. This result is not surprising given that Cer(d18:1) constitute membrane lipids which coalesce to membrane rafts, which are capable of substantial modulation of cellular function (van Blitterswijk et al. 2003). Further, the contribution of the recycling pathway to remodelling of peripherally derived ceramide is not yet known and certainly, the uptake and metabolism of circulating Cer(d18:1) into the brain has yet to be quantified or studied.

Further research will be needed to understand the biological role of circulating sphingolipids in neurodegenerative diseases. If circulating ceramides influence the brain, it is important to distinguish the direct effects on CSF sphingolipids, amyloid-beta and tau (Mielke *et al.* 2014, Fonteh *et al.* 2015) and potential for inducing cerebrovasculature dysfunction, a major risk factor for neurodegenerative diseases (Takechi *et al.* 2010). The cerebral capillaries provides an active interface between blood and the brain and thus, regulates the passage of molecules into and out of the brain, including lipids. Sphingolipid signalling in cerebral endothelial cells appear to be highly selective of protective versus dysfunctional states (Testai *et al.* 2014, van Doorn et al. 2012, Prager *et al.* 2015). Furthermore, accumulation of ceramides has been shown to induce endothelial dysfunction in the periphery (Zhang *et al.* 2012).

A number of limitations applied to this study: (1) only one internal standard per sphingolipid class was used. It is assumed that minimal differences in response factor exist with changes in length and saturation of the fatty acyl chains. (2) Small, but biologically significant, changes in some brain lipids may not be observable with the current sample sizes. A priori power analysis was performed to achieve a sensitivity sufficient for modest changes in Cer(d18:1), based on prior publications. (3) Not all known sphingolipid classes were examined. Certainly, the number of sphingolipid classes is vast, each with considerable differences in chemical and biophysical properties. For this reason,

development of LC-MS/MS protocols for simultaneous detection of all sphingolipid classes is impractical. Therefore the focus of this paper was on the major classes present and those reported in previous studies. Only male mice were used in this study. Sex differences in circulating sphingolipid levels have been reported in both animals and humans (Hammad *et al.* 2010, Mielke et al. 2015). Currently, it is not known whether these differences correspond to altered susceptibility for neurodegenerative diseases.

In summary, using an array of sphingolipid modulating agents in mice fed a SFA enriched diet, the results presented herein suggest: plasma sphingolipids are principally regulated through the de novo synthesis pathway; the HPF and CTX regulate Cer(d18:1) abundance through different pathways; plasma Cer(d18:1) concentrations do not appear to reliably predict, nor influence the abundance of Cer(d18:1) in the HPF or CTX. Collectively, this study identifies the different metabolic pathways that regulate Cer(d18:1) concentrations in plasma and the brain.

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Acknowledgements

This study was supported by the National Health and Medical Research Council of Australia. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscripts. The authors declare no conflict of interest.

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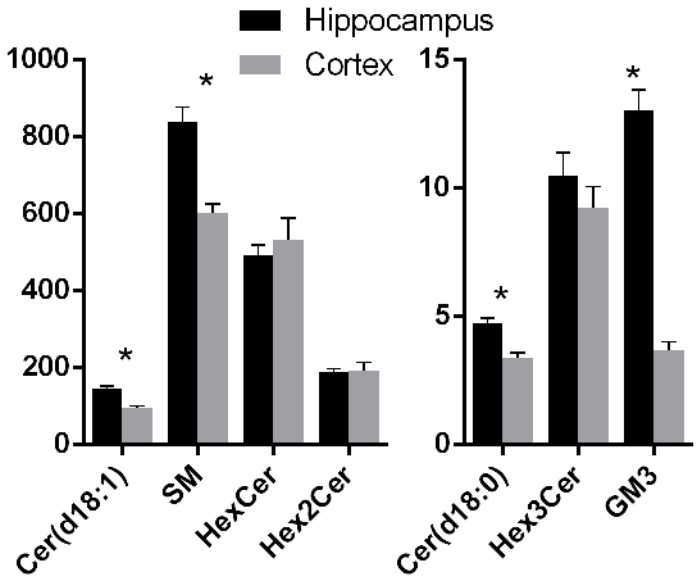
Table 1. Relative abundance of sphingolipid classes in plasma, hippocampus and cerebral cortex.

	CTRL	SFA	MYR	AMI	FTY
Plasma					
Cer(d18:1)	1.00 ± 0.14 ‡	1.62 ± 0.41 †	0.93 ± 0.12 ‡	1.53 ± 0.27 †	1.88 ± 0.60 †
Cer(d18:0)	0.13 ± 0.04 ‡	0.57 ± 0.18 †	0.34 ± 0.2 †‡	0.60 ± 0.29 †	0.5 ± 0.19 †
SM	68.3 ± 7.8	68.5 ± 20.7	60.7 ± 6 †	60.5 ± 9.8	71.2 ± 24.5
HexCer	11.5 ± 1.6	10.7 ± 2	7.49 ± 0.83 †‡	10.5 ± 2.1	12.4 ± 4.6
Hex2Cer	0.33 ± 0.06	0.31 ± 0.04	0.42 ± 0.09 †‡	0.30 ± 0.03	0.31 ± 0.05
Hex3Cer	0.17 ± 0.03	0.16 ± 0.04	0.17 ± 0.03	0.16 ± 0.03	0.18 ± 0.03
GM3	0.12 ± 0.05	0.12 ± 0.06	0.25 ± 0.06 †‡	0.13 ± 0.05	0.17 ± 0.08
Hippocampus					
Cer(d18:1)	145 ± 21	136 ± 25	128 ± 28	129 ± 29	113 ± 21 †‡
Cer(d18:0)	4.71 ± 0.7	4.63 ± 0.8	4.53 ± 1.2	4.48 ± 0.9	4.28 ± 1.0
SM	839 ± 118	789 ± 83	786 ± 62	809 ± 70	812 ± 104
HexCer	493 ± 81	535 ± 58	493 ± 102	540 ± 102	525 ± 114
Hex2Cer	189 ± 25	210 ± 20	217 ± 39	224 ± 45 †	216 ± 36
Hex3Cer	10.5 ± 2.9	10.6 ± 1.9	11.2 ± 2.5	11.2 ± 2.5	9.9 ± 2.8
GM3	13 ± 2.6	12.7 ± 2.0	12.0 ± 2.5	13 ± 2	13 ± 3.1
Cerebral Cortex					
Cer(d18:1)	94.1 ± 17.2	87.8 ± 15.6	76.3 ± 12.4 †	81.5 ± 16.0	98.1 ± 21
Cer(d18:0)	3.37 ± 0.62	3.45 ± 0.54	3.21 ± 0.73	2.94 ± 0.64	3.5 ± 0.62

SM	601 ± 76	613 ± 50	584 ± 78	628 ± 101	615 ± 84
HexCer	532 ± 176	484 ± 84	564 ± 115	478 ± 166	447 ± 89
Hex2Cer	192 ± 70	186 ± 37	214 ± 35	176 ± 57	170 ± 43
Hex3Cer	9.21 ± 2.65	9.1 ± 2.05	9.99 ± 1.45	9.57 ± 2.73	8.23 ± 1.33
GM3	3.67 ± 1.04	3.77 ± 0.8	4.08 ± 1.69	3.98 ± 1.2	4.49 ± 0.97

562 Mean ± SD. Plasma, pmol/µL; Hippocampus, pmol/mg brain; Cerebral cortex, pmol/mg brain. CTRL,
563 control diet fed group; SFA, saturated fat enriched diet fed group; MYR, myriocin treated mice; AMI,
564 amitriptyline treated mice; FTY, FTY720 treated mice; Cer(d18:1), ceramide; Cer(d18:0),
565 dihydroceramide; SM, sphingomyelin; HexCer, monohexosylceramide; Hex2Cer, dihexosylceramide;
566 Hex3Cer, trihexosylceramide; GM3, G_{M3} ganglioside. N=9-10 per group. † $P < 0.05$ vs CTRL. ‡ $P < 0.05$
567 vs SFA.



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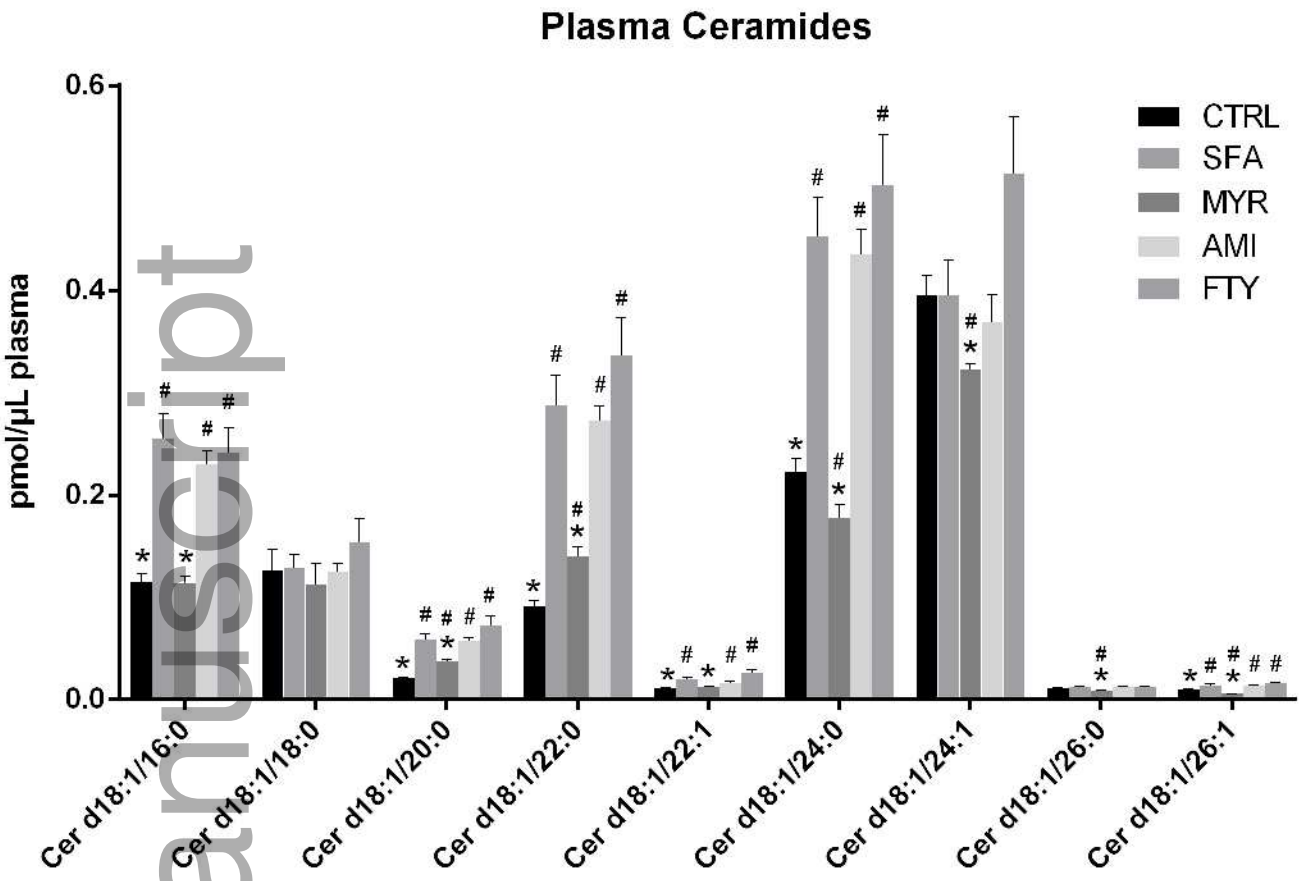

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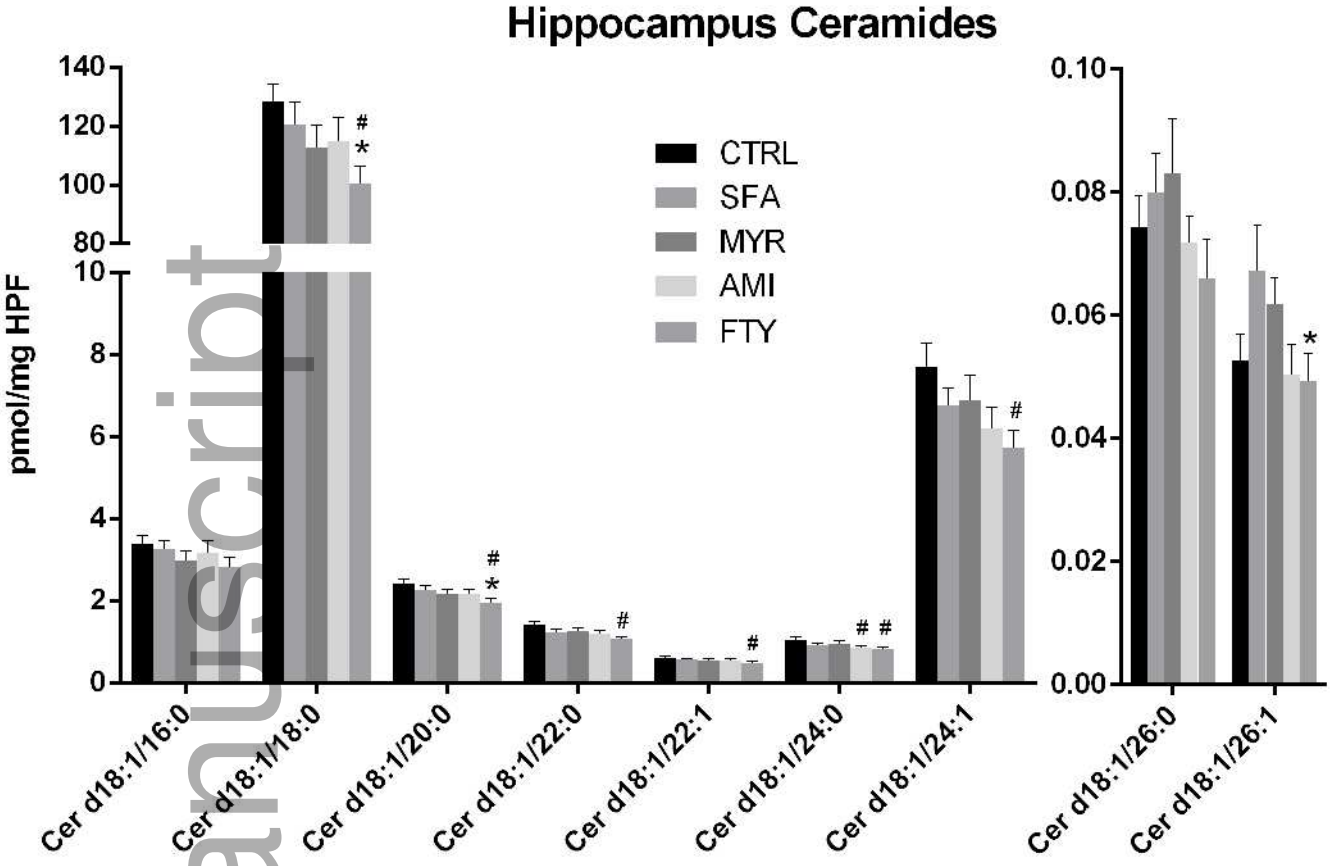

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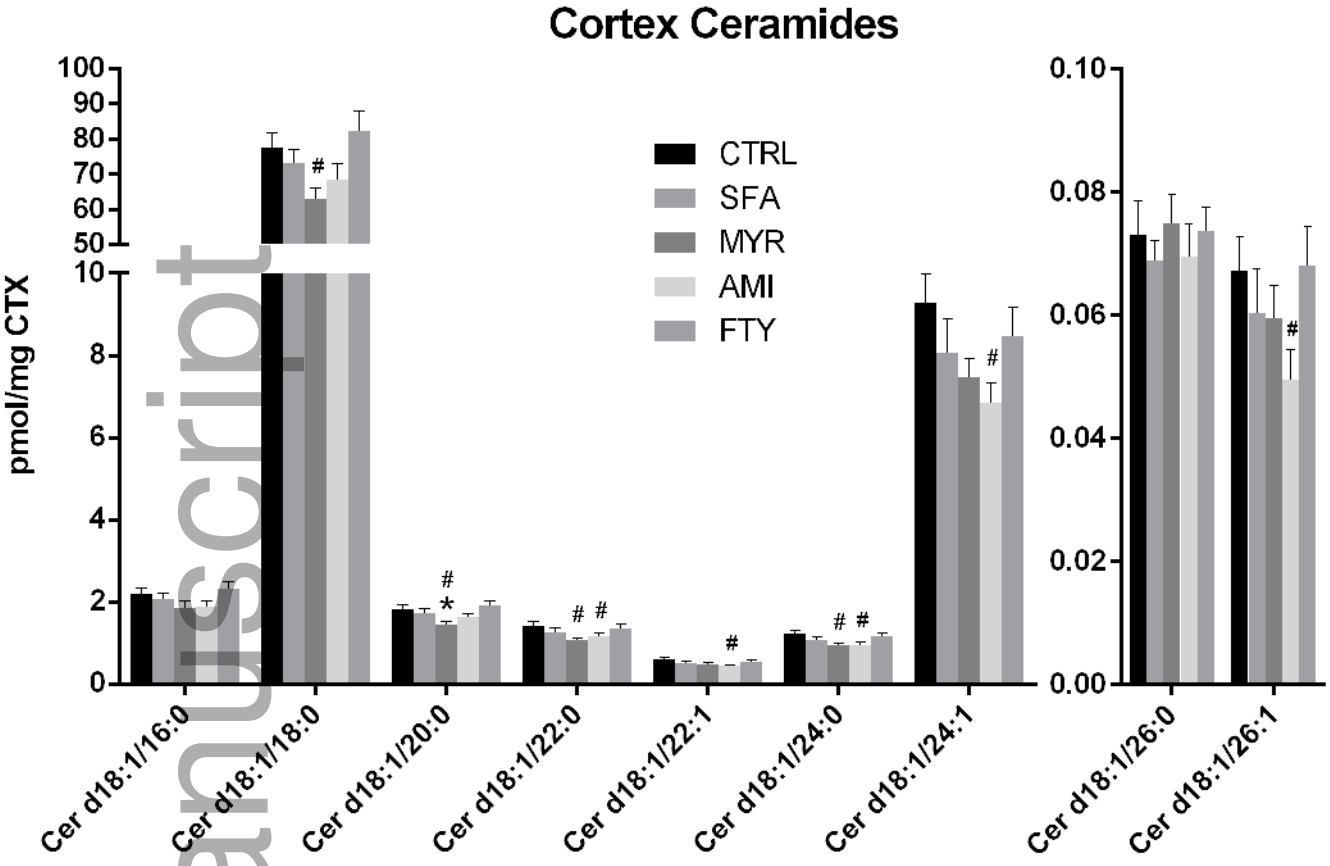

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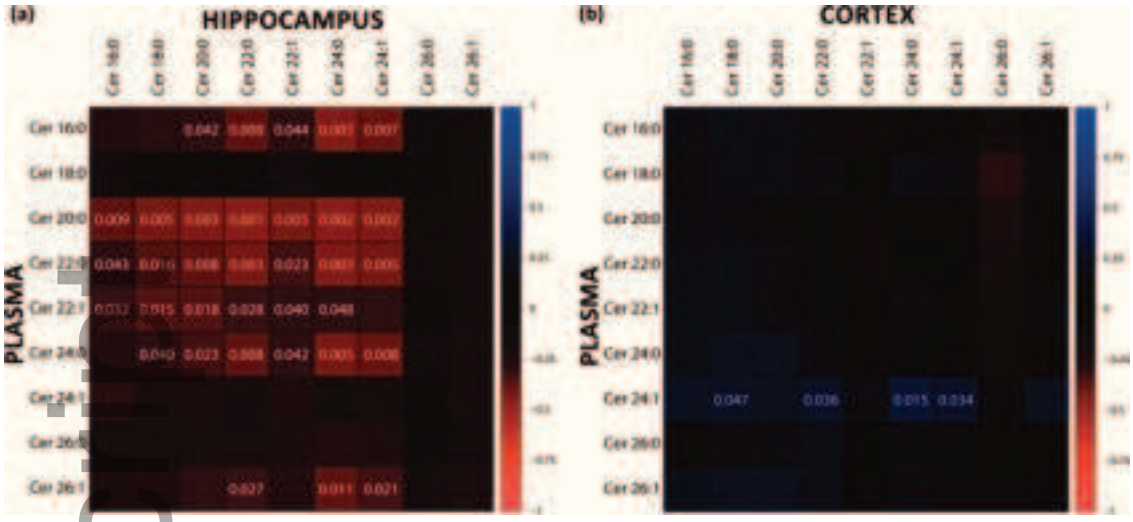

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