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Differential regulation of sphingomyelin synthesis and catabolism in oligodendrocytes and neurons

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Abstract

Neurons (both primary cultures of 3-day rat hippocampal neurons and embryonic chick neurons) rapidly converted exogenous NBD-sphingomyelin (SM) to NBD-Cer but only slowly converted NBD-Cer to NBD-SM. This was confirmed by demonstrating low *in vitro* sphingomyelin synthase (SMS) and high sphingomyelinase (SMase) activity in neurons. Similar results were observed in a human neuroblastoma cell line (LA-N-5). In contrast, primary cultures of 3-day-old rat oligodendrocytes only slowly converted NBD-SM to NBD-Cer but rapidly converted NBD-Cer to NBD-SM. This difference was confirmed by high *in vitro* SMS and low SMase activity in neonatal rat oligodendrocytes. Similar results were observed in a human oligodendroglioma cell line. Mass-Spectrometric analyses confirmed that neurons had a low SM/Cer ratio of (1.5 : 1) whereas oligodendroglia had a high SM/Cer ratio (9 : 1). Differences were also confirmed by [³H]palmitate-labeling of ceramide, which was higher in neurons compared with oligodendrocytes. Stable transfection of human oligodendroglioma cells with neutral SMase, which enhanced the conversion of NBD-SM to NBD-Cer and increased cell death, whereas transfection with SMS1 or SMS2 enhanced conversion of NBD-Cer to NBD-SM and was somewhat protective against cell death. Thus, SMS rather than SMases may be more important for sphingolipid homeostasis in oligodendrocytes, whereas the reverse may be true for neurons.

Keywords

embryonic chick neurons; fluorescent NBD-sphingolipids; neonatal rat hippocampal neurons; neonatal rat oligodendrocytes; neurotumor cell lines; sphingomyelin synthase

Several forms of neurodegenerative disease are currently being treated with sphingolipid-modifying drugs such as the serine:palmitoyltransferase inhibitor myriocin, ceramide: UDP-glucose:glucosyltransferase (CGIT) inhibitors [*N*-butyldeoxynojirimycin and *D*-threo-1-3', 4'-ethylenedioxyphenyl-2-palmitoylamino-3-pyrrolidinopropanol (Et-DO-P4)],

sphingomyelinase (SMase) inhibitors (desiprimine and GW4869), and antioxidants, as well as sphingolipid-modifying enzyme replacement, gene, and stem cell therapy (Haughey *et al.* 2004; Jeyakumar *et al.* 2005). However, we know little of the factors that regulate sphingolipid homeostasis in different brain cell types, or even if such differences exist. Knowledge of sphingolipid compositional differences between brain cell types and the ability of sphingolipids to be taken up by cells has permitted the diagnosis of neural-specific lysosomal storage diseases in fibroblasts. Such cells do not synthesize brain-specific lipids (reviewed by Buccolerio and Futerman 2003; Tettamanti 2004) but can accumulate exogenously added lipids if they lack the specific lysosomal hydrolase. Thus, the addition of exogenous oligodendrocyte-specific sulfolactosylceramide (sulfatide) to fibroblasts results in accumulation of sulfatide if the patient has metachromatic leukodystrophy (arylsulfatase-A deficiency; Kihara *et al.* 1983). Similarly, the feeding of exogenous neuron-specific GM2-ganglioside to fibroblasts from patients with β -hexosaminidase A deficiency (Tay—Sachs disease) leads to its accumulation and the ability to diagnose variant forms of Tay—Sachs (Dawson *et al.* 1986). The role of serum lipoproteins in supplying cells with glycosphingolipids is well documented (Dawson *et al.* 1976) and GM1-deficient C6-glioma cells can acquire functional cholera toxin binding sites (GM1-ganglioside) by exposure to fetal calf serum (Pacuszka and Fishman 1991).

We therefore set out to determine if fluorescent-labeled sphingolipids could be an alternative to the use of radioisotope-labeled lipids to study ceramide homeostasis in cells of CNS origin and compared hippocampal neurons and oligodendrocytes isolated from the same animals to see if we could demonstrate differences in the main pathways of ceramide-sphingomyelin (SM)—glycolipid interconversions. We focused on ceramide as ceramide levels are typically elevated in neurodegenerating brains (e.g. Puranam *et al.* 1997) and ceramide levels have been shown to increase in both neonatal rat oligodendrocytes (NRO) (Testai *et al.* 2004a) and cultured embryonic chick neurons (ECN) (Weisner and Dawson 1996; Venkataraman and Futerman 2000; Yu *et al.* 2000) undergoing cell death. In addition, ceramide is a target for many drugs currently being used to ameliorate neurodegenerative disease in children with Gaucher, Krabbe, Tay—Sachs, and Niemann—Pick disease (Jeyakumar *et al.* 2005). Ceramide has been implicated as either an effector or an enhancer in both the death receptor (cytokine) and stress (BCL₂ family) pathways which lead to cell death (Goswami and Dawson 2000; Chalfant *et al.* 2002) but in some circumstances, e.g. in fetal neurons at low concentrations (Irie and Hirabayashi 1999), it may be protective. Ceramide is clearly unique in increasing mitochondrial membrane permeability and cytochrome *c* release (Siskind *et al.* 2006) and many studies implicate a neutral sphingomyelinase (NSMase2) as the source of this ceramide in apoptosis (e.g. Kolesnick and Hannun 1999; Wiesner *et al.* 1997; Marchesini *et al.* 2003; Lee *et al.* 2004; Testai *et al.* 2004a; Chen *et al.* 2006). Thus, pro- β amyloid peptide activation of NSMase activates c-Jun NH₂ terminal kinase and BH₃-only pro-apoptotic protein DP5 pro-apoptotic pathways in oligodendrocytes (Chen *et al.* 2006). A pivotal role for acid sphingomyelinase (ASMase) in generating proapoptotic ceramide has also been observed in neuronal cerebral ischemia (Yu *et al.* 2000) and cytokine action (Gulbins and Kolesnick 2002), but additional pathways may also be involved (Riboni *et al.* 2000).

As metabolites of ceramide such as sphingosine-1-phosphate (S-1-P) can be bioprotective (Chalfant and Spiegel 2005), the enzymes which regulate ceramide levels must themselves be highly regulated as they connect pathways with antagonistic properties. It is therefore important to understand the role of ceramide metabolizing pathways in neurons and glia as different brain cell types may respond differently to drugs used to treat neurological disorders such as brain tumors, neurodegenerative diseases, and psychiatric disorders. This is especially important because of the current interest in treating lysosomal storage diseases by restricting the synthesis of sphingolipids (Jeyakumar *et al.* 2005).

Other important sphingolipid pathways in nervous tissue must include *de novo* synthesis of dihydrosphingosine, dihydro-Cer and then ceramide (Fig. 1) from serine and palmitoyl-CoA, as mutations in the serine palmitoyltransferase gene result in hereditary peripheral neuropathy (Dawkins *et al.* 2001). Ceramide is catabolized by ceramidases [both acid (lysosomal) and neutral (mitochondrial)] (El Bawab *et al.* 2002; Sawai *et al.* 2005) to sphingosine, and then by sphingosine kinases action (SK1 or SK2) to S-1-P. S-1-P signals through at least five different G protein-coupled receptors in the CNS and deletion of SK is embryologically lethal in mice (Chalfant and Spiegel 2005). However, over-expression of SK1 in lung cells actually generated dihydrosphingosine-1-phosphate (DHS-1-P) rather than S-1-P (Berdyshev *et al.* 2006), suggesting increasing complexity and the possibility of cell-type heterogeneity in sphingolipid metabolism. Ceramide can also be directly phosphorylated to Cer-1-phosphate (Chalfant and Spiegel 2005; Sawai *et al.* 2005) and this is important in the nervous system as mutations in the ceramide kinase gene lead to accumulation of Cer and premature apoptotic cell death in the brain (Tuson *et al.* 2004).

The recent cloning of two sphingomyelin synthases (SMS1 and 2) which convert Cer into SM (Huitema *et al.* 2004; Yamaoka *et al.* 2004) has suggested additional ways in which ceramide levels could be regulated in the brain and elsewhere (Fig. 1). In addition to the ceramide—SM cycle (Kolesnick and Hannun 1999), ceramide is converted by ceramide:UDP-galactose:galactosyltransferase to GalCer (in oligodendrocytes) as part of myelination (Dupree and Popko 1999) and by CGIT to form GlcCer [mainly in neurons and astrocytes (ASTs)] as shown in Fig. 1. GlcCer is the core of more than 200 glycolipids, and many of the gangliosides have specific biological activities (Furukawa *et al.* 2002; Simpson *et al.* 2004). If CGIT is conditionally deleted in neural cells, ceramide increases and major neurological symptoms result (reduced axonal branching, cerebellar dysfunction, etc.) soon after birth (Jennemann *et al.* 2005). In contrast, CGIT is often activated in many tumors (including brain tumors), thus reducing the levels of proapoptotic-Cer (Liu *et al.* 2001).

Despite the obvious importance of ceramide to brain health (Haughey *et al.* 2004) and this diversity of metabolic pathways capable of regulating ceramide levels there is relatively little information available as to how ceramide levels are actually regulated in brain or even in intact cells derived from the nervous system. We now report that fluorescent (NBD-labeled) sphingolipid analogs can be used to show major differences between oligodendrocytes and neurons and that these differences are supported by *in vitro* enzyme assays, gene transfection studies as well as mass-spectrometric (MS) analysis and isotope-labeling studies.

Materials and methods

Chemicals and drugs

C6-NBD-SM, C6-NBD-ceramide, and C₂-ceramide were purchased from Avanti and Matreya Inc. (Pleasant Gap, PA, USA). Staurosporine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Hoechst 33285 were from Sigma (St Louis, MO, USA). Silica gel LHP-K high performance thin-layer chromatography (HPTLC) plates were from Whatman (Clifton, NJ, USA) and the protein assay kit was from BioRad (Hercules, CA, USA). Hexamethylumbelliferyl-phosphorylcholine was purchased from Moscerdam Substrates, Amsterdam, Holland. Chloroform, methanol, and acetic acid used for HPTLC were ACS grade from Fisher Scientific (Pittsburgh, PA, USA). Glucosyltransferase inhibitor, D-threo-1-3',4'-ethylenedioxyphenyl-2-palmitoylamino-3-pyrrolidinopropanol, was a generous gift from Dr J. Shayman, University of Michigan School of Med (Ann Arbor, MI, USA). Constructs containing the genes for SMS1 and SMS2 were generous gifts from Dr Christophe Poirier (University of Chicago, Chicago, IL, USA).

Cell culture

Neurotumor cell lines—Human oligodendroglioma (HOG) cells were grown in monolayers on 100-mm tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% gentamycin. HOG cells were originally derived from a HOG and express some characteristics of immature oligodendrocytes (Post and Dawson 1992; Dawson *et al.* 1993; Buntinx *et al.* 2003). Human neuroblastoma-derived LA-N-5 cells were cultured under identical conditions as described previously (Cho and Dawson 2000).

The vector pCMV 3X-FLAG (Sigma) was used to over-express NSMase2 in HOG cells. Stable clones were selected on the basis of neomycin (G418-sulfate) resistance (Goswami *et al.* 2005b) and cultured in the presence of neomycin as described above. SMS1 and 2 catalyze the conversion of ceramide and phosphatidylcholine (PC) to SM and diacylglycerol (DAG). To study ceramide/sphingolipid homeostasis we cloned SMS1 (mouse cDNA using an *EcoR1* site) into expression vector pCAGGS, which has a chicken c-actin promoter with a rabbit β -globin polyA tail and SMS2 into pCMV sport6 vector (Invitrogen Inc., Carlsbad, CA, USA) (Goswami *et al.* 2005b). Following transfection by electroporation, stable clones were established using neomycin selection as described previously for NSMase2 over-expression (Goswami *et al.* 2005b). All clones were assayed for SMS activity by *in vitro* assay as described below.

Primary cultures

Neonatal (P3) rat hippocampal neurons—Briefly, hippocampi were dissected from 10 to 12 anesthetized decapitated 3-day-old rats, sectioned (400- μ m thickness) and incubated in oxygenated, pH-buffered saline (Marks *et al.* 2000). Tissue was treated with proteases and mechanically triturated to yield a cell suspension which was centrifuged through an iodixanol density gradient for 15 min. The pellet was treated with DNase and cells plated on poly-lysine-coated six-well Co-star plates in 5% oxygen/5% CO₂ in nitrogen in Neurobasal culture medium (Life Technologies) supplemented with B-27 (2 mL/100 mL; Life Technologies, Grand Island, NY, USA), 2-mercaptoethanol (0.14 mM), NaCl (35 mM), glutamine (0.5 mM), penicillin (1000 U/mL), and streptomycin (0.1 mg/mL) (Marks *et al.* 2000).

Neonatal (P3) rat oligodendrocytes and astrocytes—Neonatal rat oligodendrocytes were isolated from cerebral hemispheres of the same rats used for neonatal rat hippocampal neurons (NRH) culture by the Bottenstein (1986) modification of the method of McCarthy and de Vellis (1980). The first shake is carried out at 7 days and subsequently at 1-week intervals with shakes 2, 3, and 4 giving the highest yield of 98% pure AST and microglia-free oligodendrocytes (NRO). All cultures were differentiated for 6 days prior to use (Scurlock and Dawson 1999; Testai *et al.* 2004a). Rat ASTs were isolated by trypsinization of the bed layer (following oligodendrocyte removal by shaking) and culture in DMEM supplemented by 10% fetal bovine serum.

Embryonic chick neurons—Embryonic chick neurons were derived from freshly dissected embryonic day 7 cerebral hemispheres and were cultured on polylysine-coated plates as described previously (Mangoura *et al.* 1988; Dawson 2000; Krueger *et al.* 2001). ECN were cultured in DMEM for 6 days prior to addition of drugs, during which time they formed aggregates and extended interconnecting neurites as shown in Fig. 2.

Labeling studies with NBD-sphingolipids

NBD-Cer (formula weight 576 compared with 565 for the natural C18:0 ceramide), NBD-SM, and other NBD-sphingolipids were used at a concentration of 1 mg/mL in ethanol. They were added to cell cultures to achieve a final concentration of 1 μ g/mL. Typical labeling experiments

were carried out in 60-mm Petri dishes, containing 2 mL of serum-free medium, for 24 h. Drugs were either given for 24 h before labeling, at the same time, or after 24 h (e.g. when using agents to induce cell death). Cells (0.5×10^6 /60 mm plate) were harvested and washed three times with phosphate-buffered saline, and the pellet was extracted with methanol (1 mL). Following addition of chloroform (2 mL) and 0.01 N HCl (0.6 mL) the upper phase was discarded and the lower phase was evaporated to dryness under nitrogen (Wiesner *et al.* 1997; Kilkus *et al.* 2003). Lipids were applied to HPTLC plates (10 × 10 cm; LHP-K TLC plates) and developed in chloroform : methanol : glacial acetic acid : water (70 : 25 : 8.8 : 4.5 v/v). Bands were visualized by fluorography and quantified with a BioRad Chemi-Doc XRS scanner over the linear range, using Quantity One software. This digitizes the chemi-fluorescent image for quantification. Some of the digital HPTLC images presented have been overexposed to reveal minor components so that in some instances there may not appear to be differences between the major bands. Cells were also labeled with [^3H]palmitate and lipids isolated as described previously (Kilkus *et al.* 2003).

Sphingomyelin synthase assay

Harvested cells were homogenized and sonicated in 10 mM Tris—HCl buffer pH 7.4 (0.2 mL) containing EDTA (20 mM) and no Mg^{2+} . Extracts (50 μg protein) were incubated with NBD-Cer (1 μg) and PC (20 μg) as phosphorylcholine donor as previously described (Taguchi *et al.* 2004; Yamaoka *et al.* 2004), and drugs as indicated. The fluorescent products were isolated by HPTLC using the solvent system chloroform : methanol : acetic acid : water (70 : 25 : 8.8 : 4.5 v/v) (Kilkus *et al.* 2003). Bands were visualized by fluorography and quantified with a BioRad Chemi-Doc XRS scanner over the linear range using Quantity One software.

Sphingomyelinase assays

Acid SMase and NSMase activity was determined with the fluorimetric substrate hexamethylumbelliferyl-phosphorylcholine as described previously (Testai *et al.* 2004a).

Ceramide:UDP-glucose:glucosyltransferase assay

Ceramide:UDP-glucose:glucosyltransferase was assayed by adding UDP-glucose (500 μM) to the extract instead of PC, prior to incubation as described previously (Yamaoka *et al.* 2004).

Cell viability assays

Cells were plated in 24-well culture plates for MTT assay, which was carried out as described previously (Wiesner *et al.* 1997). DNA fragmentation was determined as the soluble DNA secreted into the culture medium following induction of apoptosis (Dawson *et al.* 2004), using calf DNA as the standard and determining the fluorescence from DNA binding to Hoechst 33258, as described previously (Dawson *et al.* 2004).

LC-MS/MS analysis

Lipids from 1×10^6 cells were extracted, using 0.01 N HCl (to prevent formation of phosphatidylmethanol) and lipid phosphorus was determined as described previously (Berdyshev *et al.* 2006). C_{17} -Ceramide, C_{17} -sphingosine and C_{17} -S-1-P were added at this stage as internal standards. Samples were evaporated under nitrogen and treated with 100 μL pyridine and 50 μL acetic anhydride at 40°C for 20 min prior to liquid chromatography-MS/MS analysis. An AP1-4000 Triple Quadrupole Mass-Spectrometer (Applied Biosystems, Foster City, CA, USA) interfaced with an Agilent 1100 liquid chromatograph and autosampler was used for analysis of S-1-P, DHS-1-P, sphingosine 3-keto-dihydrosphingosine (3KDHS), ceramide and dihydroceramide (Berdyshev *et al.* 2006).

Shotgun mass-spectrometry

Lipids from the pellets of approximately 1×10^6 cells were extracted by a modified procedure of Bligh and Dyer in the presence of 50 mM LiCl in the aqueous phase and internal standards for lipid analyses (Cheng *et al.* 2006). The internal standards used include 16:0–16:0 GPEtn, 14:1–14:1 GPCho, 15:0–15:0 GPSer, 15:0–15:0 GPGro, 17:0 ceramide, 16:0 sulfatide, d₃₅-18:0 GalCer, and 12:0 SM. The lipid extracts were concentrated under nonoxidizing conditions, filtered, and re-extracted with 20 mM LiCl as previously described (Cheng *et al.* 2006). Electrospray ionization/MS analyses were carried out with a ThermoElectron TSQ Quantum Ultra (Thermo Electron Corp., San Jose, CA, USA) using 2 min. signal averaging in the profile mode (Cheng *et al.* 2006). For anionic lipids (e.g., anionic phospholipids and sulfatide), the negative-ion mode was used. Addition of a small amount of LiOH in methanol prior to infusion facilitates the analysis of choline glycerophospholipids and SMs as their lithium adducts in the positive-ion mode and permits the analysis of ethanolamine glycerophospholipids in the negative-ion mode (Han *et al.* 2006). Individual lipid molecular species were identified by multidimensional MS as previously described (Han and Gross 2005a,b).

Statistical analysis

The results expressed as HPTLC analyses are all representative of experiments performed at least three times. The *in vitro* assay results are based on experiments run in triplicate at least twice. Statistical analyses were performed by Student's *t*-test and results were considered statistically significant when $p < 0.05$.

Results

NBD-Cer and NBD-SM are metabolized at different rates in primary cultures of hippocampal neurons and oligodendrocytes cultured from the same 3-day-old rats

We previously showed that exogenous NBD-SM was taken up by HOG cells and that the conversion to NBD-Cer was stimulated by both staurosporine treatment and over-expression of NSMase2 (Goswami *et al.* 2005b). This and other data suggested that exogenously added NBD-SM is metabolized similarly to endogenous SM as determined by other techniques (radioisotope-labeling, MS, etc.). We therefore compared the metabolism of exogenous NBD-Cer and NBD-SM in rat hippocampal neurons and oligodendrocytes isolated from the same 3-day-old rat brains (Scurlock and Dawson 1999; Marks *et al.* 2000) (representative TLCs are shown in Fig. 2). Primary neuronal (NRH and ECN) cultures slowly (10–15%) converted NBD-Cer to NBD-SM (Fig. 2, lanes 1 and 3), compared with NRO, which rapidly converted > 80% of NBD-Cer to NBD-SM in 24 h (Fig. 2, lane 5). The NBD-glycosylceramides were GlcCer in NRH and ECN and GalCer in NRO as expected, based on the prevalence of GalCer in myelin and previous analysis of neurons and oligodendrocytes. In contrast NBD-Cer was rapidly converted to SM by NRH and ECN (50–60%) compared with NRO (20%). ECN and NRH showed low SM/Cer ratios (Fig. 2) whereas oligodendrocytes showed high SM/Cer ratios. The relative amounts of NBD-lipids extracted from the cells/mg of protein were comparable but the relative amounts of NBD-Cer and NBD-SM were quite different.

To summarize Fig. 2, when NBD-Cer was added to neurons, the NBD-SM : NBD-Cer ratio was (1 : 10) ECN and (1 : 5) NRH but 50- to 100-fold higher in oligodendrocytes (10 : 1). Conversely, when NBD-SM was added to neurons the NBD-SM : NBD-Cer ratio was 5-fold higher in neurons ECN and NRH (1 : 1) compared with oligodendrocytes (1 : 5). We then carried out several different types of studies to confirm and explain these large differences between neuronal cells and oligodendrocytes.

SMS activity is higher in NRO than in ECN

The schematic diagram (Fig. 1) shows the two major pathways for interconversion of Cer and SM (SMases and SMSs) and the NBD-labeling studies suggested that SMS (in which PC is the phosphocholine donor) should be more active in NRO than in NRH or ECN. We confirmed these differences by *in vitro* SMS assay. SMS1 is believed to be primarily in Golgi whereas a second enzyme, SMS2, is found primarily in the plasma membrane (Huitema *et al.* 2004). Homogenates of both ECN and NRO (presumably containing both SMS1 and 2 activity) were incubated with NBD-Cer and SMS activity was stimulated by the exogenous phosphocholine donor PC. The enhanced conversion of NBD-Cer to NBD-SM in NRO was reflected in the > 4-fold higher activity of SMS in oligodendrocytes compared with neurons (Fig. 3). As a control we added UDP-glucose to the same homogenates in the presence of NBD-Cer (Fig. 3) and showed that it enhanced the formation of NBD-GlcCer in ECN much more than in NRO, which express mainly ceramide:UDP-galactose:galactosyltransferase.

ASMase and NSMase activities are higher in NRH than in NRO or rat astrocytes

In vitro assay of both ASMase and NSMase in extracts of NRH, NRO, and AST (as a control) prepared from the same 3-day-old rats showed a more than 2-fold increase in specific activity of both SMases in the (NRH) compared with either glial cell type (Fig. 4a and b), thus further supporting the NBD-labeling data.

Neurotumor cell lines metabolize NBD-SM and Cer at different rates

Human oligodendroglioma cells took up exogenous NBD-Cer and metabolized 70% of it to NBD-SM and NBD-GlcCer in 24 h, whereas only 16% of exogenous NBD-SM was converted to NBD-Cer in 24 h (Fig. 5). Human neuroblastoma cell line (LA-N-5), selected because it expresses a number of neuronal-specific proteins such as growth associated protein-43 (Cho and Dawson 2000), took up exogenous NBD-Cer but only converted 12% to NBD-SM in 24 h, compared with 46% conversion of exogenous NBD-SM to NBD-Cer (Fig. 5). In summary, when cells were fed NBD-Cer, the NBD-SM : Cer ratio was > 10-fold higher in HOG compared with LA-N-5 whereas when cells were fed NBD-SM, the NBD-SM : Cer ratio was > 4-fold higher in LA-N-5 than in HOG. This agrees with the differences previously observed (Fig. 2) between primary oligodendrocytes (NRO) and neuronal cultures (NRH and ECN).

Over-expression of sphingomyelin synthases (SMS1 and 2) increases SM levels in HOG cells and protects against cell death

Human oligodendroglioma cells which stably over-expressed NSMase2 (Goswami *et al.* 2005b), SMS1 or 2 were compared with wild-type HOG for their ability to convert exogenous NBD-Cer to NBD-SM in 24 h. NSMase2 over-expression resulted in a 50% decrease in NBD-SM levels, an increase in NBD-Cer and increased NBD-GlcCer (Fig. 6), together with increased susceptibility to cell death as described previously (Goswami *et al.* 2005b). In contrast, SMS1 and 2 over-expression, although less dramatic, resulted in increased NBD-SM (Fig. 6) and protection against cell death (Fig. 7b and c). The *in vitro* SMS assay showed a > 10-fold increase over basal SMS activity in neomycin-selected stable HOG clones which over-expressed SMS1 [Fig. 7a (2) compared with wild-type (1)]. In intact cells, this translated only into a 50% increase in NBD-SM levels, which probably reflects the complexities of multiple pathways and lipid transport systems in intact cells, as shown in Fig. 1. Cells over-expressing SMS1 showed increased resistance to staurosporine-induced cell death (50% death requiring 15 nM compared with 10 nM in non-transfected cells). This was verified by both DNA fragmentation (Fig. 7b) and the MTT mitochondrial viability assays (Fig. 7c).

NBD-Sphingolipids and their metabolites in culture medium

The same pattern of NBD-sphingolipids was observed in both cells and culture medium. Thus, both NBD-Cer and NBD-SM could be re-isolated from the culture medium when NBD-SM was added to ECN cultures (Fig. 8a and b, lanes 1–3) (reflecting high SM to Cer conversion in the ECN cells) whereas essentially only NBD-Cer could be isolated from the culture medium when NBD-Cer was added (Fig. 8a and b, lanes 4–6) reflecting the low level of Cer to SM conversion in ECN cells. The presence of NBD-ceramide in the culture media could represent non-uptake, or ceramide ‘shedding,’ as observed when we added NBD-SM to the cells.

LC-MS/MS and shotgun mass-spectrometric analysis validates the use of NBD lipids and confirms the high Cer/SM ratio in neurons compared with oligodendrocytes

We have previously reported increased conversion of NBD-SM to NBD-Cer by intact HOG cells when we over-expressed NSMase2 or treated with staurosporine (Goswami *et al.* 2005b). We confirmed this 50% increase in Cer by LC-MS/MS (Table 1) but Cer levels were found to be normal in HOG cells over-expressing SMS1 or 2 (Table 1). There was little difference in the ratio of Cer to dihydro-Cer when NSMase, SMS1, or SMS2 were over-expressed but a > 2-fold increase in the ratio of ceramide to free C18 sphingosine was observed in all three over-expressing cells (Table 1).

If the NBD-Cer/SM labeling studies were physiologically valid, we reasoned that MS analysis should reveal a much higher SM/Cer ratio in neurons compared with oligodendrocytes. The shotgun MS approach (Han *et al.* 2006) was used to measure Cer and SM and it can be seen from Fig. 9a—c that the SM/Cer ratio in ECN was 1.5 whereas in NRO and HOG cells it was > 8.0. The shotgun MS analysis approach (Han *et al.* 2006) also confirmed data obtained using NBD-Cer as the reporter lipid, in that glucosyltransferase inhibitor Et-DO-P4 reduced the SM/Cer ratio by 25% (Fig. 9a and b). *De novo* synthesis inhibitor myriocin decreased the SM/Cer ratio only 10% whereas fumonisins B1 increased it 2-fold (Fig. 9).

A more detailed analysis of sphingolipid metabolites requires a pre-step of HPLC separation (Berdyshev *et al.* 2006). LC-MS/MS analysis revealed that dihydrosphingosine forms (C18-sphinganine, sphinganine-1-phosphate, and dihydroceramide) were minor components in both vector alone and NSMase2-transfected HOG cells and that although ceramide was elevated by NSMase2, other sphingosines were reduced by 50%. However, when we transfected both vector alone and NSMase2 over-expressing HOG cells with SK1, DHS-1-P was synthesized > 100-fold in preference to S-1-P, becoming equal in concentration to ceramide (1.5 pmol/nmol compared with 1.8 pmol/nmol lipid phosphorus) (Table 2). The over-expression of SK1 reduced Cer levels by 10% and only increased S-1-P levels 2-fold. This is in agreement with previous SK1 over-expression studies in a human pulmonary artery endothelial cells lung cell line (Berdyshev *et al.* 2006). SK1 over-expression reduced sphingosine levels by a third (to 0.06 pmol/nmol lipid phosphorus) but for the first time we were able to detect the presence of 3KDHS (Table 2), a proposed intermediate in the formation of dihydrosphingosine from serine and palmitoyl-CoA (3KDHS in Fig. 1).

To further confirm the ceramide differences between neurons and oligodendrocytes we compared ceramide synthesis levels in hippocampal neurons (NRH) and oligodendrocytes (NRO) isolated from the same 3-day-old rats and labeled with [³H]palmitate for 24 h (Fig. 10). We isolated detergent-resistant membranes (or Rafts) by sucrose-density gradient as previously described (Kilkus *et al.* 2003; Goswami *et al.* 2005b), and found that the amount of ceramide synthesized/mg protein by NRH was 3-fold more than that synthesized by NRO. Thus, the Cer : SM ratio was 0.34 in NRO and 0.91 in NRH, whereas the ratio of Cer to cholesterol was essentially the same, 0.27 compared with 0.34 in NRH (Fig. 10).

Discussion

NBD-sphingolipids have a truncated fatty acid component (C6-NBD) compared with the endogenous C_{16–24} fatty acids found in sphingolipids but they have a slightly higher molecular weight (576 vs. 565), are still hydrophobic and show strong affinity for cell membranes. We present evidence that NBD-SM and NBD-Cer can be used to show major differences in the metabolism of these lipids by primary cultures of neurons and oligodendrocytes. We show that we can modify the metabolism of NBD-sphingolipids by activating or over-expressing their major metabolizing enzymes (NSMase2, SMS1, and SMS2) and that the differences observed with exogenous fluorescent sphingolipids are supported by differences observed from MS analysis and isotope labeling. Our main conclusion is that basal ceramide levels are much higher in neurons than in oligodendroglia whereas SM levels are comparable and that this relates to ASMase, NSMase, and SMS enzyme activity. A HOG cell line gave results similar to the NRO and a human neuroblastoma cell line (LA-N-5) gave results very similar to both NRH and ECN. ECN share many characteristics with neonatal rodent neurons (Mangoura *et al.* 1988; Dawson 2000; Krueger *et al.* 2000), have comparable lipid composition to both rat cerebellar granule cells and embryonic mouse neurons (Yu *et al.* 2000; Brann *et al.* 2002), and show apoptosis in response to C2-ceramide concentrations similar to that reported for mammalian neuronal cell lines (Weisner and Dawson 1996; Dawson 2000). Our results suggest that the observed neuronal—oligodendrocyte differences occur in cells from different species, different developmental stages and in their derived tumor cells and that this should be considered when neuro-therapeutic options are being considered.

Our findings show that exogenous NBD-sphingolipids can be used to look at neural pathways with all the advantages of high sensitivity (by virtue of their fluorescence), but with no requirement for specialized services or disposal (as with radioisotopes). Further, their use has highlighted an under-appreciated pathway of SMS from ceramide which is low in neurons (where levels are high) and high in oligodendrocytes where ceramide is low. Clearly, NSMase plays a major role in regulating ceramide levels and is higher in neurons than in oligodendrocytes, thus contributing to the higher basal ceramide level in neurons. We therefore tried to evaluate if SMS also plays a role in maintaining ceramide homeostasis. Over-expression of SMS1 or 2 in HOG cells only minimally reduced Cer but increased SM by 30%, as well as conferring some protection against cell death, so SMS is a factor in Cer homeostasis. NSMase2 is clearly induced by stress in all cell types (e.g. Goswami *et al.* 2005b) and therefore has a key role in neurodegeneration but the role of SMS needs further study. The use of NBD lipids could help in the pre-clinical evaluation of sphingolipid-modifying drugs such as those currently being used to treat sphingolipidoses (Jeyakumar *et al.* 2005), the treatment of cancer by elevating cellular ceramide levels and in the treatment of reperfusion-induced brain injury (Shao *et al.* 2007).

We focused on NBD-ceramide as α -erythro-ceramide has been implicated in many aspects of cell death, probably through activation of a phosphatase (Chalfant *et al.* 2002) which degrades anti-apoptotic phospho-Akt (PK-B) (Goswami *et al.* 1999) leading to mitochondrial release of cytochrome c regulated by the BCL₂ protein family (Chalfant *et al.* 2002). Ceramide (Cer) also activates a range of pro-apoptotic, calcium-dependent kinases (Kolesnick and Hannun 1999), the stress-activated protein kinase/c-Jun NH₂ terminal kinase pathway (Verheij *et al.* 1996; Goswami *et al.* 1999), and up regulates pro-apoptotic genes, at least in dopaminergic neurons (Unschuld *et al.* 2006). By using NBD-sphingolipids and quantitative HPTLC, we were able to study sphingolipid metabolism pathways in detail and start to understand how Cer levels, and the above pathways, are differentially regulated in individual brain cell types.

Fluorescent NBD-glycosphingolipids (as well as their Boron difluoride dipyromethane dye-labeled analogs) have been previously reported to be taken up and metabolized by

oligodendrocytes from spinal cord (Giudici *et al.* 1992) and brain (deVries *et al.* 1993). Most of the incorporation of NBD-ceramide was into the Golgi complex, and some Golgi outstations in the neurites/processes. More recent studies by Pagano and others have shown internalization of NBD sphingolipids by clathrin-independent caveolar (Raft)-related and dynamin-dependent mechanisms into rat hippocampal neurons (Babia *et al.* 2001) and a variety of other cells (Singh *et al.* 2003; Sharma *et al.* 2004; Tomas *et al.* 2004). Less polar NBD-sphingolipids such as glucosylceramide, ceramide, and SM, the focus of this study, appeared to be internalized from the plasma membrane by a combination of endocytic and non-endocytic mechanisms, but initially all end up in the Golgi complex rather than the lysosomes (Babia *et al.* 2001). The mechanism of transport appears to be independent of species or the stage of differentiation but little is known of the precise fate of the individual C6-NBD-lipids in neural cells as all the above studies followed the fate of the NBD-sphingolipids qualitatively by microscopy.

Our major novel finding is the unexpected high activity of SMS and low activity of NSMase in oligodendrocytes, which results in rapid NBD-Cer to SM conversion and keeps ceramide levels low and SM and galactosylceramide levels high. SMS1 and 2 convert Cer into SM (using PC as a phosphocholine donor) and generating DAG as the other product. This reaction is catalyzed by a PC-SM transacylase (Wanner *et al.* 2004) with the plasma membrane form (SMS1) a likely candidate as it was able to restore SM synthesis and cell growth in SMS-negative lymphoid cells (Yamaoka *et al.* 2004). The high level of SMS activity in NRO was comparable to that seen in oligodendroblast-derived HOG cells (Post and Dawson 1992; Dawson *et al.* 1993; Buntinx *et al.* 2003). The importance of the pathway in NRO was emphasized by inhibiting CGIT (Abe *et al.* 2000) and showing elevation of both NBD-Cer and NBD-SM.

In contrast, SMS activity was low and NSMase activity high in neurons and NBD-Cer was poorly converted to NBD-SM, whereas NBD-SM was rapidly converted to NBD-Cer. The *in vitro* assay of ASMase and NSMase activity showed a 2-fold higher activity in NRH compared with rat glia (NRO or ASTs) from the same brains. These results, together with the *in vitro* SMS enzyme data agree with the finding of much higher basal ceramide levels in neurons compared with oligodendrocytes, suggesting that different brain cell types regulate ceramide levels by different mechanisms. However, there did not appear to be overall ceramide compositional difference between human gray and white matter (Han *et al.* 2002) so the *in vivo* situation may be more complex.

Maintenance of Cer-SM homeostasis in neural cells could control the balance between life (DAG) and death (Cer) and is obviously critical at all times of development. For example, growth factor deprivation of human natural killer cells induced activation of ASMase, inhibition of CGIT, and inhibition of SMS (Taguchi *et al.* 2004). All of these resulted in increased ceramide together with reduced DAG levels and cell death (Riboni *et al.* 2000; Karakashian *et al.* 2004). Growth factor rescue was blocked by either adding C₂-Cer or inhibiting PI3kinase to lower phosphatidyl-myoinositol 3,4,5-triphosphate [Ptd(Ins3,4,5)P₃] levels. We have previously shown that both inorganic phosphate (20 mM) and Ptd(Ins3,4,5)P₃ are potent non-competitive inhibitors of ASMase (Testai *et al.* 2004b). Therefore, lowering Ptd(Ins3,4,5)P₃ should activate ASMase (Testai *et al.* 2004b) and elevate ceramide. Similarly, pro-apoptotic tumor necrosis factor- α was found to inhibit SM synthesis from ceramide and activate the conversion of SM to ceramide in rhabdomyosarcoma cells (Riboni *et al.*, 2000), thereby increasing both ceramide and cell death. In contrast, proliferative agents such as basic fibroblast growth factor lowered ceramide levels by activating SM synthesis (presumably through SMS activation) and inhibiting SMases (presumably NSMase2) (Goswami *et al.* 2005a, Goswami *et al.* 2005b). Thus, the regulation of SM-Cer homeostasis by growth factors is an important neural cell function necessary for survival, and the use of exogenous NBD-

sphingolipids can help quantify these changes and lead to better understanding of neurotrophic factor-sphingolipid interactions.

There is growing *in vivo* evidence to support the idea that regulation of ceramide is critical to normal brain function. Thus, mutations in ASMase produce devastating neurovisceral storage of SM (Niemann—Pick Type A disease) and ballooning of neurons (Kuemmel *et al.* 1997). In ASMase (–/–) mice, the degeneration of Purkinje cells and other neurons is early and complete, there is only minor SM storage in oligodendrocytes (Kuemmel *et al.* 1997). Axonal dystrophy is pronounced but there is little dysmyelination, supporting our observation (based on NBD-SM catabolism) that SMase is more active in neurons than in oligodendrocytes. In contrast, NSMase2 (–/–) mice show developmental changes in the brain (Stoffel *et al.* 2005) and osteogenesis imperfecta (Aubin *et al.* 2005). Only the stress response appears to be compromised. Mutations in acid ceramidase lead to ceramide accumulation in lysosomes (Farber's Lipogranulomatosis) and neurodegeneration (Futerman and Van Meer 2004). Although elevated lysosomal ceramide does not result in increased apoptosis, it is possible that failure to convert ceramide to the bioactive S-1-P could have pathological consequences. Thus in *Drosophila*, the slug-a-bed gene ceramidase controls presynaptic terminal sphingolipid composition to regulate vesicle fusion, trafficking, and synaptic function (Rohrbough *et al.* 2004) suggesting that regulation of ceramide catabolism must be critical for normal nervous system function in many species and phyla.

In therapeutic endeavors, CGIT inhibitors such as *N*-butyldeoxynojirimycin have been used to attempt to lower glycolipid substrate levels in the neurological forms of Gaucher disease and juvenile Tay—Sachs disease (Jeyakumar *et al.* 2005) but it is clear from our studies that such inhibitors also elevate ceramide more dramatically in NRO than in neurons. Thus, the regulation of ceramide levels in cells is a complex process, offering many targets for therapeutic intervention. We propose that loading NBD-sphingolipids lipids can be used to quantitatively study the interactions of these critical sphingolipids in primary cell cultures to understand basic mechanisms of neural cell responses to survival factors and to better understand the mechanism of action of neurotherapeutic drugs.

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Abbreviations used

3KDHS, 3-keto-dihydrosphingosine
 ASMase, acid sphingomyelinase
 AST, astrocyte
 Cer, ceramide (2-*N*-acylsphingosine)
 CGIT, ceramide:UDP-glucose:glucosyltransferase
 DAG, diacylglycerol
 DHS-1-P, dihydrosphingosine-1-phosphate
 DMEM, Dulbecco's modified Eagle's medium
 ECN, embryonic chick neurons
 Et-DO-P4, *D*-threo-1–3', 4'-ethylenedioxyphenyl-2-palmitoylamino-3-pyrrolidinopropanol
 HOG, human oligodendroglioma
 HPTLC, high performance TLC
 MS, mass-spectrometry
 MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
 NBD-Cer, C6-NBD (7-nitrobenz-2-oxa-1, 3-diazol-4-yl) *D*-erythro-C18-sphingosine

NRH, neonatal rat hippocampal neurons
 NRO, neonatal rat oligodendrocytes
 NSMase2, neutral sphingomyelinase
 PC, phosphatidylcholine
 PtdIns(3,4,5)P₃, phosphatidyl-myoinositol 3,4,5-triphosphate
 S-1-P, sphingosine-1-phosphate
 SK, sphingosine kinase
 SM, sphingomyelin
 SMase, sphingomyelinase
 SMS, sphingomyelin synthase

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Fig. 1.

Potential metabolic pathways for NBD-sphingolipid metabolism major pathways involving ceramide are highlighted. Right-hand side pathways (lipids in boxes) can be followed with NBD-lipids whereas mass-spectrometry is required for left-hand side e.g. S-1-P. SMS1 and SMS2 are sphingomyelin synthases. Sphingomyelin (SM) is catabolized to ceramide by both lysosomal (ASMase) and neutral membrane (NSMase2) sphingomyelinases. SPT is serine palmitoyltransferase, ceramide:UDP-galactose:galactosyltransferase (CGT), and CGIT are Cer UDP-Gal and Glc-glycosyltransferases, respectively, ACerase is lysosomal and NCerase is neutral ceramidase. CERKL is ceramide kinase, SK1 and SK2 are sphingosine kinases 1 and 2, and 3KDHS is 3-keto-dihydrosphingosine.

Fig. 2.

TLC analysis of the fate of NBD-Cer and NBD-SM HPTLC of isolated sphingolipids showing three major components, NBD-Cer (Cer), NBD-Glc/GalCer (GlyCer), and NBD-SM (SM) following treatment of 2×10^6 cells with NBD-lipids for 18 h. Appearance of cells used for actual studies in shown in the three panels: embryonic chick neurons (ECN), neonatal rat hippocampal neurons (NRH), and neonatal rat oligodendrocytes (NRO) are all 200 $\times$ magnification. HPTLC: ECN: lane 1 (fed NBD-Cer), lane 2 (fed NBD-SM), NRH, lane 3 (fed NBD-Cer), lane 4 (fed NBD-SM), NRO lane 5 (fed NBD-Cer), and lane 6 (fed NBD-SM). Cells were treated, isolated, and characterized as described in the text. The percent conversion of added lipid to metabolic product (e.g. in lane 1, the percent conversion of NBD-Cer to NBD-SM) is shown along with the SM/Cer ratio.

Fig. 3.

Sphingomyelin synthase activity is higher in oligodendrocyte than in neuronal cell extracts
Panel (a): Quantification of NBD-SM formed *in vitro* from NBD-Cer in the absence (none) or presence (phosphatidylcholine; PC) of exogenous PC showing increased activity in NRO compared with ECN. Panel (b): Quantification of NBD-GlcCer formed *in vitro* from NBD-Cer in the absence (none) or presence (UDP-Glc) of exogenous UDP-glucose (+) showing increased activity in ECN compared with NRO.

Fig. 4. Sphingomyelinase activities in primary cultures isolated from 3-day-old rat brains (a) ASMase activity in NRO, astrocytes (AST), and NRH. (b) NSMase activity in NRO, AST, and NRH. Enzyme activities were assayed as described in the text. Note that the ASMase/NSMase ratio is (10 : 1).

Percent recovered in cell				
	LAN-5		HOG	
	NBD-Cer	NBD-SM	NBD-Cer	NBD-SM
NBD-Cer	57	46	30	16
NBD-GlcCer	32	20	36	13
NBD-SM	12	34	34	72

Fig. 5.

NBD-Cer is metabolized more actively and NBD-SM less actively, by human oligodendrogloma-derived cells (HOG) compared with human neuroblastoma-derived cells (LA-N-5). Cells were cultured and treated for 24 h as described in the text. The HPTLC plates were chromatographed in chloroform : methanol : glacial acetic acid : water (70 : 25 : 8.8 : 4.5 v/v). Bands were visualized by fluorography and quantified with a BioRad Chemi-Doc XRS scanner, using Quantity One software. This digitizes the chemi-fluorescent image for quantification.

Lane	1	2	3	4
Cer	3200	6000	2900	3100
SM	3300	1810	4400	4600

Fig. 6.

Effect of NSMase and SMS over-expression on NBD-Cer metabolism in HOG cells
 Representative HPTLC of HOG cells showing lipids from wild-type (lane 1), NSMase2 over-expressing (lane 2), SMS1 over-expressing (lane 3), and SMS2 over-expressing (lane 4) after labeling for 24 h with NBD-Cer Lipids were extracted and quantified as described in the text. Mobility of authentic NBD-Cer, NBD-GlcCer and NBD-SM are indicated.

Fig. 7.

SMS1 over-expression: SMS *in vitro* activity and protection against cell death (a) HPTLC assay of *in vitro* SMS activity determined as described in the text. (1) Duplicate samples of wild-type plus exogenous PC; (2) Duplicate samples of over-expressed SMS1 extracts plus exogenous PC. (b) Decreased staurosporine-induced DNA fragmentation after SMS1 over-expression (open squares). (c) Decreased cell death by staurosporine after SMS1 over-expression (open squares).

Fig. 8.

The pattern of NBD-lipids in cells is reflected in the NBD-sphingolipid composition of the culture medium. (a) Fate of exogenous NBD-SM (lanes 1–3) and NBD-Cer (lanes 4–6) in cells after incubation with intact ECN cells for 24 h. (b) Fate of exogenous NBD-SM (lanes 1–3) and NBD-Cer (lanes 4–6) in culture medium after incubation with intact ECN cells for 24 h.

Fig. 9. Shotgun mass-spectrometric analysis reveals low SM/Cer ratios in cells of neuronal origin ECN (panel a) and high SM/Cer ratios in cells of oligodendroglial cell origin NRO (b) and HOG (c). GTI, CGIT inhibitor treatment; Myr, myriocin-treatment; St, staurosporine treatment; FB1, fumonis-B1 treatment. Experimental details of treatment and analysis are provided in the text.

Fig. 10.

Increased ceramide synthesis by neurons (NRH) compared with oligodendrocytes (NRO) Autoradiogram of [^3H]lipids in detergent-resistant membranes (Rafts) isolated (Kilkus *et al.* 2003) from NRH and NRO cells labeled for 24 h with [^3H]palmitate. Lipids were extracted, phosphoglycerides removed by alkaline methanolysis and cholesterol (Chol), ceramide (Cer), and Sphingomyelin (SM) separated by HPTLC as described in the text.

Table 1
LC-MS/MS mass-spectrometric sphingolipid analysis of HOG cells transfected with NSMase2, SMS1, and SMS2

Lipids	Vector only	NSMase2	SMS1	SMS2
Percent change from control				
Ceramide	100	148	110	94
Ratio of lipid species				
Cer/DihydroCer	29	24	27	27
Cer/Sphingosine	9	23	16	20

Individual lipids (including fatty acid species) were quantified by reference to an internal standard (C₁₇). Results are based on triplicate analyses. The major fatty acid species in Ceramide and dihydro-ceramide were 16:0, 24:0, and 24:1, with lesser amounts of 18:0, 22:0, and 26:1 in ceramide. HOG, human oligodendroglioma; NSMase2, neutral sphingomyelinase; SMS, sphingomyelin synthase.

Table 2

LC-MS/MS analysis of sphingolipids

Lipid ratio	Vector only		NSMase2	
	–	+SK1	–	+SK1
S-1-P/DHS-1-P	2.1	0.04	5.8	0.04
Sph/DHSph	3.2	2.7	3.6	1.8
Cer/DHCer	16	9	16	6
DH Sph/3-keto-DHS	> 1000	1.3	> 1000	1.2

Over-expression of NSMase2 and SK1 in HOG cells increases ceramide but also affects levels of sphingolipid metabolites. Results are based on triplicate analyses. 3-keto-DHS was identified by its retention time (4.7 min compared with 4.5 min for sphingosine) on HPLC. DHS-1-P, dihydrosphingosine-1-phosphate; HOG, human oligodendrogloma; MS, mass-spectrometry; SK, sphingosine kinase; NSMase2, neutral sphingomyelinase; 3-keto-DHS, 3-keto-dihydrosphingosine.