Influence of Caloric Restriction on Motor Behavior, Longevity, and Brain Lipid Composition in Sandhoff Disease Mice

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Caloric restriction (CR), which improves health and increases longevity, was studied as a therapy in a hexosaminidase β knockout mouse model of Sandhoff disease (SD), an incurable neurodegenerative disease involving accumulation of brain ganglioside GM2 and asialo-GM2 (GA2). Adult mice were fed a rodent chow diet either ad libitum (AL) or restricted to reduce body weight by 15–18% (CR). Although GM2 and GA2 were elevated, no significant differences were seen between the Hexb–/– and the Hexb+/+ mice for most brain phospholipids and cholesterol. Cerebrosides and sulfatides were reduced in the Hexb–/– mice. In addition, rotorod performance was significantly worse in the Hexb–/– mice than in the Hexb+/+ mice. CR, which decreased circulating glucose and elevated ketone bodies, significantly improved rotorod performance and extended longevity in the Hexb–/– mice but had no significant effect on brain lipid composition or on cytoplasmic neuronal vacuoles. The expression of CD68 and F4/80 was significantly less in the CR-fed than in the AL-fed Hexb–/– mice. We suggest that the CR delays disease progression in SD and possibly in other ganglioside storage diseases through anti-inflammatory mechanisms. © 2006 Wiley-Liss, Inc.

Key words: inflammation; ganglioside; ketone; rotorod; glucose; neurodegeneration

Sandhoff disease (SD) is an incurable glycosphingolipid (GSL) storage disorder that involves primarily the storage of ganglioside GM2 and its asialo derivative GA2 in brain and visceral tissues. The gangliosides are a family of sialic acid–containing glycosphingolipids (GSL) enriched in neuronal membranes and are involved in cell–cell and cell–matrix interactions (Ledeen, 1983). GM2 storage in SD occurs largely in neuronal lysosomes and leads to progressive neurodegeneration and brain dysfunction (Neufeld, 1991; Gravel et al., 1995). Reduction of cerebrosides and sulfatides, associated with dysmyelination, also accompanies GM2 accumulation (Sandhoff et al., 1971). SD is inherited as an autosomal recessive disorder as a result of pathogenic mutations in the lysosomal hexosaminidase β subunit, which contributes to the quaternary structure of both hexosaminidase A (Hexa) and hexosaminidase B (Hexb) isoenzymes. Patients with SD lack both the Hexa and Hexb isoenzymes, unlike patients with Tay-Sachs disease, who lack Hexa. The failure to catabolize GM2 to GM3 in lysosomes is characteristic of both disorders (Sandhoff and Kolter, 2003).

A mouse model of SD, deficient in Hexb activity, was generated by using homologous recombination and embryonic stem cell technology (Sango et al., 1995). These mice mimic the pathological, biochemical, and clinical abnormalities of the human disease and can be used to explore new therapeutic approaches. Similarly to SD patients, the Hexb-deficient mice have a rapidly progressive neurodegenerative course that can facilitate associations among neurochemical pathology, behavior, and lipid storage (Sango et al., 1995; Phaneuf et al., 1996; Jeyakumar et al., 2003). We recently showed that these mice accumulate GM2 and GA2 in the brain as early as postnatal day 5 (p-5), thus mimicking the neurochemical features of the infantile disease form (Baek et al., 2004). However, Hexb-deficient mice are phenotypically indistinguishable from normal mice until adult ages, unlike infantile-onset SD patients, in whom ganglioside accumulation leads to behavioral and developmental abnormalities within the first few years of life. The Hexb-deficient mice have been used to evaluate therapies for reducing GSL storage to include bone marrow transplantation (BMT); enzyme replacement therapy (ERT); substrate reduction therapy (SRT), which aims to decrease the rate of GSL biosynthesis to counterbalance the impaired rate of catabolism; gene therapy; and, most recently, stem cell therapy (Norflus et al., 2003).
Neurodegeneration in SD involves microglial activation, macrophage infiltration, and oxidative damage (Wada et al., 2000). These changes are also associated with increased apoptosis and expression of inflammatory markers, including CD68 and F4/80 (Jeyakumar et al., 2003). It is not clear, however, the extent to which the neurodegeneration in SD results from pathological GSL accumulation or from neuroinflammation that accompanies this accumulation. Recent studies in SD mice show that nonsteroidal anti-inflammatory drugs (NSAIDs) increase life span and improve motor behavior associated with disease progression, suggesting that inflammation by itself contributes to disease pathogenesis (Jeyakumar et al., 2004). Furthermore, the combination of NSAIDs with the National Institutes of Health (NIH), Propionibacterium acnes (Gronblad et al., 2000). The neuroprotective effects of CR occur from aging by decreasing the production of reactive oxygen species and glial activation (Morgan et al., 1999; Duan et al., 2003; Koubova and Guarner, 2003). CR also has neuroprotective effects in rodent models of neurodegenerative diseases (Mattson, 2003). CR is produced from a total dietary restriction and differs from acute fasting or starvation in that CR reduces total caloric energy intake without causing anorexia or malnutrition (Mantis et al., 2003; Veech, 2004). CR is produced from a total dietary restriction and differs from acute fasting or starvation in that CR reduces total caloric energy intake without causing anorexia or malnutrition (Mantis et al., 2003; Veech, 2004). CR diminishes neuroinflammation and oxidative stress that occur from aging by decreasing the production of reactive oxygen species and glial activation (Morgan et al., 1999; Lee et al., 2000). The neuroprotective effects of CR likely result from reduced glucose levels and elevated ketone bodies (β-OHB), which reduces oxygen free radicals and increases the ΔG’ of ATP hydrolysis (Greene et al., 2003; Mantis et al., 2003; Veech, 2004).

In this study, we investigated the effects of CR in a mouse model of SD (Hexb+/– mice) during a period of severe disease progression (100–125 days of age). Our results show that CR significantly improved motor performance and extended longevity in the Hexb+/– mice but had no major effects on brain lipid composition or cytoplasmic neuronal vacuoles. Moreover, CR reduced expression of the inflammatory markers CD68 and F4/80. We suggest that CR may be a palliative therapy for SD and other ganglioside storage diseases. A preliminary report of these findings was recently published in abstract form (Denny et al., 2005).

MATERIALS AND METHODS

Mice

Mice heterozygous for the Hexb gene (Hexb+/–) were provided by Dr. Richard Proia (National Institutes of Health, Bethesda, MD). The mice were derived by homologous recombination and embryonic stem cell technology (Sango et al., 1998; Jeyakumar et al., 1999, 2004; Kasperzyk et al., 2004; Baek et al., 2005; Afri et al., 2005).

Caloric Restriction

All mice received Prolab RMH 3000 chow (LabDiet, Richmond, IN). The diet contains a balance of mouse nutritional ingredients and delivers 4.4 kcal g−1 gross energy, where fat, carbohydrate, protein, and fiber comprised 55, 520, 225, and 45 g kg−1 of the diet, respectively. After a 10-day pretrial period, the mice were placed into two groups (n = 4–5 mice/group) in which the average body weight of each Hexb+/– group was similar. At 100 days of age, the mice were assigned to either a control group that was fed standard lab chow ad libitum (AL) or an experimental group that was fed the same diet in restricted amounts (CR). Each mouse in the CR group served as its own control for body weight reduction. Based on food intake and body weight during the pretrial period, food in the CR-fed group was gradually reduced until each mouse achieved the target weight reduction of 15–18%. This was comparable to an approximate 30–35% reduction in calories per day. Body weight was used as an independent variable to reduce the variability among individual mice for the effects of caloric restriction (Pugh et al., 1999; Greene et al., 2001; Mantis et al., 2004). The target body weight reduction for all CR-fed Hexb+/– mice was reached in approximately 8 days.

The mice in the AL-fed group received 80 g of food in the hopper every 2 days between 2 PM and 3 PM as in the pretrial period. Daily food intake was estimated as the difference in food weight after 2 days divided by 2. Weighed food pellets were dropped directly into each cage for the CR-fed Hexb+/– mice and for the AL-fed Hexb+/– mice that developed motor deficiency as the study progressed.

Measurement of Plasma Glucose and β-Hydroxybutyrate

Mice were anesthetized with isoflurane (Halocarbon Laboratories, River Edge, NJ) and euthanized by exsanguination, involving collection of blood from the heart in heparinized tubes. The blood was centrifuged at 6,000g for 10 min, the plasma was collected, and aliquots were stored at −80°C until analysis. Plasma glucose concentration was measured in a spectrophotometer using the Stanbio Enzymatic Glucose Procedure (Stanbio). The ketone body β-hydroxybutyrate (β-OHB) was
measured enzymatically in plasma with a modification of the Williamson et al. (1962) procedure.

**Tissue Processing**

After sacrifice by cardiac puncture at 125 days of age, brains were collected for lipid, protein, and histological analysis. The right hemisphere was weighed and stored at –80°C. A portion of this hemisphere was then lyophilized for lipid analysis. The remaining portion was used for RNA and protein determination. The left hemisphere was stored in formalin (Sigma, St. Louis, MO) at 4°C for histological analysis.

**Rotorod**

A rotordor apparatus, consisting of a knurled dowel fixed 10 cm above Sani-Chip bedding, was used to measure motor coordination and balance. After a 3-day pretrial training period, mice were assessed for motor behavior every 5 days from 100 to 125 days of age. Mice were placed on the rotating dowel at speeds of 20, 30, 40, 52, and 60 rpm, indicating the start time for the trial. A 30-sec interval was allowed between the two trials at a given speed, and a 2-min interval was given between different speeds. The maximal time allowed on the bar for each trial was 60 sec. The trial was terminated when the mouse fell off the bar or at 60 sec. The Hexb−/− mice, often clung or wrapped around the bar as a consequence of declining ambulatory ability due to disease progression. Whereas the time on bar may be dependent on motor coordination and balance, the bar wrapping behavior may be more dependent on strength and endurance.

**Lipid Isolation, Purification, and Quantification**

Total lipids were isolated and purified from lyophilized brain tissue by using modifications of previously described procedures (Seyfried et al., 1978; Hauser et al., 2004; Kasperzyk et al., 2004). Neutral and acidic lipids were separated by using DEAE-Sephadex (A-25; Pharmacia Biotech, Uppsala, Sweden) column chromatography as previously described (Macala et al., 1983; Seyfried et al., 1984; Kasperzyk et al., 2005). The total lipid extract, suspended in solvent A (CHCl3:CH3OH:H2O, 30:60:8 by vol), was then applied to a DEAE-Sephadex column (1.2 ml bed volume) that had been equilibrated with solvent A. The column was washed twice with 20 ml of solvent A, and the entire neutral lipid fraction, consisting of the initial eluent plus washes, was collected. This fraction contained cholesterol, phosphatidylcholine, phosphatidylethanolamine, plasmalogens, ceramide, sphingomyelin, cerebrosides, and asialo–GM2 (GA2). The acidic lipids were then eluted from the column with less hydrophobic acidic lipids to include free fatty acids, cardiolipin, phosphatidylinositol, phosphatidic acid, and sulfatides. The gangliosides were isolated and purified from other acidic lipids and analyzed by using the resorcinol assay as we previously described (Kasperzyk et al., 2005).

**High-Performance Thin-Layer Chromatography**

All lipids were analyzed qualitatively by high-performance thin-layer chromatograph (HPTLC) according to previously described methods (Ando et al., 1978; Seyfried et al., 1978; Macala et al., 1983; Kasperzyk et al., 2005). Lipids were spotted on 10- × 20-cm Silica gel 60 HPTLC plates (E. Merck, Darmstadt, Germany) using a Camag Linomat III auto-TLC spotter (Camag Scientific Inc., Wilmington, NC). To enhance precision, an internal standard (oleoyl alcohol) was added to the neutral and acidic lipid standards and samples as previously described (Macala et al., 1983; Kasperzyk et al., 2005). Purified lipid standards were purchased from Matreya Inc. (Pleasant Gap, PA) or Sigma (St. Louis, MO) or were a gift from Dr. Robert Yu (Medical College of Georgia, Augusta, GA). The HPTLC plates were sprayed with either the resorcinol-HCl reagent or the orcinol-H2SO4 reagent and heated at 95°C for 30 min to visualize gangliosides or GA2, respectively (Kasperzyk et al., 2005). For neutral or acidic phospholipids, the plates were developed to a height of either 4.5 cm or 6 cm, respectively, with chloroform:methanol:acetic acid:formic acid:water (35:15:6:2.1 by vol), then developed to the top with hexane:diisopropyl ether:acetic acid (65:35:2 by vol) as previously described (Macala et al., 1983; Seyfried et al., 1984). Neutral and acidic lipids were visualized by charring with 3% cupric acetate in 8% phosphoric acid solution, followed by heating in an oven at 160–170°C for 7 min. The percentage distribution of the individual lipid bands was determined by scanning the plates on a Personal Densitometer SI with ImageQuant software (Molecular Dynamics, Sunnyvale, CA) for gangliosides, acidic lipids, and neutral lipids or on a ScanMaker 4800 with ScanWizard5 V7.00 software (Microtek, Carson, CA) for GA2.

**Histology**

The formalin-fixed left hemispheres were embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin & eosin (H&E), Luxol fast blue (LFB), and periodic acid Schiff (PAS) at the Harvard University Rodent Histopathology Core Facility (Boston, MA). Slides were examined by using a Zeiss Axioplane 2 Imaging universal microscope and a Spot Insight 4MP Fireware Color 3-shot digital camera and Spot software.

**Immunohistochemistry**

CD68 and F4/80 immunohistochemistry was analyzed via methods similar to those we previously described (Mukherjee et al., 1999, 2002). Briefly, brain tissue sections analyzed for CD68 were placed in 10 mM sodium citrate buffer, pH 6.0, at 95°C for 10 min. Slides were allowed to cool in the buffer for approximately 20 min and then washed. The sections were quenched with 0.3% H2O2–methanol for 30 min and then blocked with 10% normal donkey serum in 100 ml of 0.01 M phosphate and 0.9% sodium chloride (pH 7.4) with 1.0 g of bovine serum albumin and 0.1 ml Tween 20 (PBA buffer). The sections were then treated with an antibody to CD68 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA), followed by a biotin–SP-conjugated AffiniPure donkey anti-goat IgG (H + L) at 1:100 dilution (Jackson Immunoresearch Laboratories, West Grove, PA). The sections were next treated with avidin–biotin complex, followed by a 3,3′-diaminobenzidine as substrate for staining according to the manufacturer’s directions (Vectastain Elite ABC kit; Vector, Burlingame, CA). The sections were rinsed three times with PBS (0.01 M phosphate buffer with 0.9% NaCl), counterstained with Harris-modified hematoxylin with acetic acid
was performed to obtain optimal primer annealing temperatures for CD68 and F4/80, respectively. Gradient PCR from nucleotides 1,777 to 2,271. Amplicons were confirmed by treated with avidin-biotin complex, followed by a 3,3’-diaminobenzidine as substrate for staining according to the manufacturer’s directions (Vectastain Elite ABC kit). The sections were rinsed three times with PBS (0.01 M phosphate buffer with 0.9% NaCl), counterstained with methyl green (Vector), and mounted. A corresponding tissue section without primary antibody served as the negative control.

Semiquantitative RT-PCR

Total RNA was isolated from brain tissue with Trizol Reagent (Invitrogen, La Jolla, CA), following the manufacturer’s protocol. Spectrophotometric measurements at 260 and 280 nm determined RNA concentration and purity. Single-strand cDNA was synthesized from total RNA (3 µg) by using oligo (dT) primers (Promega, Madison, WI) in a 20-µl reaction with Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Promega) according to the manufacturer’s protocol. cDNA was used for PCR amplification of the 544-, 514-, and 497-bp regions of the CD68, β-actin, and F4/80 genes, respectively. The CD68 primers, based on Genbank NM_009853, amplified the region from nucleotides 95 to 638 (forward 5’-CAT CCT TCA CGA TGA CAC CTA CAG-3’ and reverse 5’-CTC TGA TGT AGG TCC TGT TGT AAT C-3’). The β-actin primers (forward 5’-TGT GAT GGT GGG AAT GGG TCA G-3’ and reverse 5’-TTT GAT GTC ACG CAC GAT TTC C-3’; based on GenBank NM_007393) amplified the region from nucleotides 207 to 719. The F4/80 primers (forward 5’-CTT GTC TCC TGG AAC-3’ and reverse 5’-GTG CAG CAT CTG GAT GGT GCG C-3’; based on GenBank X93328) amplified the region from nucleotides 1,777 to 2,271. Amplicons were confirmed by sequence analysis, and Genbank numbers are DQ167574 and DQ167573 for CD68 and F4/80, respectively. Gradient PCR was performed to obtain optimal primer annealing temperatures (65°C, 58°C, and 61°C for CD68, β-actin, and F4/80, respectively). To determine the linear range for the amplification reaction, PCR was performed at increasing cycle numbers, and optimal PCR cycle number was determined to be 27 cycles, 22 cycles, and 27 cycles for CD68, β-actin, and F4/80, respectively. PCR amplification of CD68, β-actin, and F4/80 was performed with Taq DNA polymerase (Promega) with the following protocol: initial denaturation at 95°C for 2 min; followed by 27, 22, or 27 cycles, respectively; denaturation at 94°C for 1 min; annealing at 65°C, 58°C, and 61°C, respectively, for 25 sec; and extension at 72°C for 1 min. A final extension at 72°C for 5 min followed the last cycle for CD68, β-actin, and F4/80 amplifications. PCR products were separated on 0.8–1% agarose gels containing ethidium bromide, visualized with UV light, and analyzed in 1D Kodak software. RT-PCR was performed on the total RNA of each sample in the absence of reverse transcriptase to control for possible DNA contamination.

Statistical Analysis

A two-tailed t-test and the Pearson product-moment correlation were used to evaluate the significance of differences between the AL and the CR groups. In each figure, n designates the number of individual mice analyzed.

RESULTS

Hexb enzymatic activity was about 50% lower in the Hexb+/– mice than in the Hexb+/+ mice, but the mice were similar in behavior, brain lipid composition, and tissue histology. Therefore, data for the Hexb+/– mice and the Hexb+/+ mice were combined and are presented as a normal control group (Hexb+/+ mice). No adverse effects were observed in the CR-fed Hexb+/– mice. Even with reduced body weight, the CR-fed Hexb+/– mice were healthier and more active than the AL-fed Hexb+/– mice as assessed by ambulatory, grooming, and nest-building behavior (data not shown). According to the standard criteria for mice, there were no signs of vitamin or mineral deficiency observed in the CR-fed Hexb+/– mice (Hoag and Dickie, 1968). These findings are consistent with the well-recognized health benefits of mild to moderate CR in rodents (Keenan et al., 1999).

Influence of CR on Body Weight

All mice were matched for age (90 days of age) before the start of the dietary treatment. The Hexb+/– mice were also matched for body weights (~27.5 g; Fig. 1A). These weights differed somewhat from those of the Hexb+/+ mice because of variability within and between litters. Whereas body weights remained relatively stable in the AL-fed Hexb+/– mice from 100 to 125 days of age, body weights in the AL-fed Hexb–/– mice remained relatively stable until 115 days of age and then dropped significantly as a result of disease progression. In the CR-fed Hexb+/– mice, body weight declined gradually until 110 days of age and then remained stable, similar to that of the Hexb+/+ mice. These findings indicate that CR prevented the body weight decline associated with disease progression in the Hexb+/– mice. The standard error of the mean for body weight ranged from 0.230 to 2.061 from 100 to 125 days of age for all groups.

Influence of CR on Plasma Glucose and β-OHB Levels

Glucose levels were approximately 12 mM for both the AL-fed Hexb+/– and the AL-fed Hexb–/– mice but were significantly lower in the CR-fed Hexb+/– mice (Fig. 1B). Plasma β-OHB levels were approximately 0.25 mM for both the AL-fed Hexb+/– and the Hexb–/– mice but were significantly elevated in the CR-fed Hexb+/– mice (Fig. 1C). These findings are consistent with our previous studies showing that β-OHB levels are increased under CR and that circulating β-OHB levels are inversely related to circulating glucose levels (Greene et al., 2001; Mantis et al., 2004).
Influence of CR on Motor Behavior and Survival

The rotorod apparatus was used to measure motor coordination and balance. Unlike the Hexb+/? mice, the Hexb−/− mice exhibited an age-dependent deficit in motor coordination and balance. Time on bar (rotorod performance) was significantly shorter in the AL-fed Hexb−/− mice than in the AL-fed Hexb+/? mice (Fig. 1D). However, the time on bar was significantly longer in the CR-fed Hexb−/− mice than in the AL-fed Hexb−/− mice, indicating that CR improved motor behavior in this task. In contrast to the AL-fed Hexb−/− mice, whose performance deteriorated rapidly from 100 to 125 days of age, the performance in CR-fed Hexb−/− mice declined less rapidly (data not shown). The performance of the Hexb+/? mice was significantly better than that of the Hexb−/− mice at all speeds and did not change significantly over the course of the study.

Normal mice rarely wrap around the rotorod bar which indicates good motor control. Wrapping behavior was significantly greater in the AL-fed Hexb−/− mice than in the AL-fed Hexb+/? mice, indicating poor motor abilities (Fig. 1E). However, wrapping was significantly less in the CR-fed Hexb−/− mice than in the AL-fed Hexb−/− mice. In contrast to the Hexb+/? mice, which normally live for approximately 2 years, the AL-fed Hexb−/− mice died between 129 and 141 days of age. CR significantly increased survival by 9% in the Hexb−/− mice (Fig. 1F).

TABLE I. Influence of Caloric Restriction on Brain Ganglioside Content in Adult Hexb Mice

<table>
<thead>
<tr>
<th>Diet</th>
<th>Genotype</th>
<th>N</th>
<th>Brain weight (mg)</th>
<th>Water content (%)</th>
<th>Ganglioside sialic acid (μg/100 mg dry wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>+/?</td>
<td>3</td>
<td>71.9 ± 2.3</td>
<td>78.77 ± 0.26</td>
<td>458 ± 36</td>
</tr>
<tr>
<td>CR</td>
<td>−/−</td>
<td>4</td>
<td>73.6 ± 1.6</td>
<td>78.83 ± 0.27</td>
<td>732 ± 27*</td>
</tr>
<tr>
<td>AL</td>
<td>−/−</td>
<td>4</td>
<td>74.5 ± 4.3</td>
<td>77.26 ± 1.29</td>
<td>725 ± 12*</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM. N, number of independent mice analyzed.

*Significantly different from that of the control mice at p < 0.01 as determined by two-tailed t-test.

Fig. 2. HPTLC of brain gangliosides in adult Hexb mice. The amount of ganglioside sialic acid spotted per lane was equivalent to approximately 1.5 μg. The plate was developed by a single ascending run with CHCl3:CH3OH:dH2O (55:45:10 by vol) containing 0.02% CaCl2 2H2O. The bands were visualized with resorcinol-HCl spray.

Fig. 1. Influence of CR on body weight (A), glucose (B), β-hydroxybutyrate (C), rotorod performance at 40 rpm (D), wrapping behavior at 40 rpm (E), and survival (F). Values are expressed as means (±SEM) and, 4–9 mice were analyzed in each group. Asterisks indicates that the value of the CR-fed Hexb−/− mice is significantly different from that of the AL-fed Hexb−/− mice at *p < 0.05 and **p < 0.01 as determined by the two-tailed t-test.

These findings considered together indicate that CR improves motor behavior, prolongs longevity, and attenuates disease progression in the \textit{Hexb}\textsuperscript{−/−} mice.

### Influence of CR on Brain Lipids

We next determined whether the CR-induced improvements in motor behavior and survival were associated with changes in brain ganglioside content and distribution in the \textit{Hexb}\textsuperscript{−/−} mice. Total brain ganglioside concentration was significantly higher in the \textit{Hexb}\textsuperscript{−/−} mice than in the \textit{Hexb}\textsuperscript{+/?} mice (Table I). CR caused no significant changes in total brain ganglioside concentration in the \textit{Hexb}\textsuperscript{−/−} mice. In addition, CR had no significant effect on brain weight or brain water content, a general marker for brain maturation (Seyfried et al., 1978). The influence of CR on the qualitative and quantitative distribution of individual gangliosides in the \textit{Hexb}\textsuperscript{−/−} mice is shown in Figure 2 and Table II. In contrast to the \textit{Hexb}\textsuperscript{+/?} mice, in which GM2 content was present in only trace amounts, GM2 was the major ganglioside in the \textit{Hexb}\textsuperscript{−/−} mice. CR had no statistically significant effect on the content of GM2 or that of other gangliosides in the \textit{Hexb}\textsuperscript{−/−} mice. The data were also analyzed by using the Pearson product-moment correlation to determine whether changes in the GM2 content in each mouse were associated with changes in motor behavior (rotorod performance). The correlation coefficient for GM2 levels and rotorod performance was not significant (\(r = 0.024, 7\) df, \(P = 0.955\)) further supporting the results from the two-tailed \(t\)-test.

These findings indicate that the CR-induced improvement in motor behavior and survival in the \textit{Hexb}\textsuperscript{−/−} mice was not due to subtle changes in brain GM2 content.

### Table II. Influence of Caloric Restriction on Brain Ganglioside Distribution in Adult \textit{Hexb}\textsuperscript{−/−} Mice

<table>
<thead>
<tr>
<th>Diet</th>
<th>Genotype</th>
<th>N(^a)</th>
<th>GM2</th>
<th>GM1</th>
<th>GD1(a)</th>
<th>GT1(a/LD1)</th>
<th>GD1(b)</th>
<th>GT1(b)</th>
<th>GO1(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>+/?</td>
<td>3</td>
<td>60 ± 16</td>
<td>167 ± 13</td>
<td>14 ± 1.2</td>
<td>51 ± 13</td>
<td>135 ± 8</td>
<td>32 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>AL</td>
<td>−/−</td>
<td>4</td>
<td>302 ± 15*</td>
<td>52 ± 9</td>
<td>156 ± 12</td>
<td>21 ± 0.8*</td>
<td>53 ± 7</td>
<td>123 ± 7</td>
<td>27 ± 1.0</td>
</tr>
<tr>
<td>CR</td>
<td>−/−</td>
<td>4</td>
<td>282 ± 11*</td>
<td>54 ± 10</td>
<td>161 ± 18</td>
<td>21 ± 0.2*</td>
<td>55 ± 7</td>
<td>125 ± 10</td>
<td>27 ± 2.3</td>
</tr>
</tbody>
</table>

\(^a\)Values represent mean ± SEM.

\(^b\)Determined from densitometric scanning of HPTLC as shown in Figure 2.

\(*\)Significantly different from that of the \textit{Hexb}\textsuperscript{+/?} mice at \(p < 0.01\) as determined from the two-tailed \(t\)-test.

The influence of CR on the qualitative and quantitative distribution of neutral and acidic brain lipids in the \textit{Hexb}\textsuperscript{−/−} mice is shown in Figures 3 and 4 and Table III. Cerabrosides and sulfatides were significantly reduced in the AL-fed \textit{Hexb}\textsuperscript{−/−} mice when compared with the AL-fed \textit{Hexb}\textsuperscript{+/?} mice. GA2, on the other hand, was significantly elevated in the AL-fed \textit{Hexb}\textsuperscript{−/−} mice when compared with the \textit{Hexb}\textsuperscript{+/?} mice. With the exception of a slight, but significant, reduction in phosphatidylcholine (\(P < 0.05\)) in the \textit{Hexb}\textsuperscript{−/−} mice, no significant differences were observed between the \textit{Hexb}\textsuperscript{+/?} mice and the \textit{Hexb}\textsuperscript{−/−} mice for the distribution of other phospholipids or for ceramide. CR had no significant effect on the distribution of these lipids in the \textit{Hexb}\textsuperscript{−/−} mice. It is important to mention that the solvent front (SF) does not include lipids but rather slight impurities from the organic solvents used in the developing system. These findings indicate that CR improve motor behavior and longevity through marked effects on the content or distribution of major brain lipids.

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Hexb were noticeably greater in the nucleus gracilis of the AL-fed mice. Dystrophic neurons, a sign of neurodegeneration, were detected in the brains of the AL-fed mice with PAS, which also identifies cytoplasmic glycolipid material in the AL-fed mice. Sections of the cerebellum were stained with LFB to visualize lysosomal storage material (Fig. 5A). No LFB-positive staining was also associated with significant reductions in CD68 and F4/80 CNS immunostaining was noticeably less in the CR-fed Hexb–/– mice than in those of the AL-fed Hexb–/– mice (Fig. 6A,B). However, CD68 and F4/80 immunostaining was noticeably less in the CR-fed Hexb–/– mice than in the AL-fed Hexb–/– mice. The strongest staining appeared in cells with a neuronal morphology. The reduction in CD68 and F4/80 gene expression in the CR-fed Hexb–/– mice (Fig. 6C,D). These findings indicate that CR reduces expression of inflammatory markers in the brains of Hexb–/– mice.

**DISCUSSION**

CR is produced from a total restriction of dietary nutrients and differs from starvation in that CR reduces total caloric energy intake without causing anorexia or malnutrition (Tannenbaum et al., 1942, 1959; Weindruch et al., 1988; Kritchevsky, 1999; Mukherjee et al., 1999; Greene et al., 2001, 2003; Seyfried and Mukherjee, 2005). As a natural dietary therapy, CR improves health and longevity, reduces expression of inflammatory markers in the brain. CR also significantly increased survival and attenuated disease progression in SD mice that accumulated large amounts of GM2 and GA2 in the brain. CR also significantly improved motor behavior and coordination in these mice.

Recent studies indicate that inflammation is a hallmark of the pathogenesis in ganglioside storage diseases, including SD (Myerowitz et al., 2002; Jeyakumar et al., 2003). This inflammatory phenotype consists of progressive microglial activation, macrophage recruitment, and proinflammatory cytokine production in the brain (Wada et al., 2000; Jeyakumar et al., 2003). Progressive macrophage activation also precedes neuronal apoptosis and the onset of neurological deterioration (Wu and Proia, 2004). Consistent with these findings, we found that expression of

**Table III. Influence of Caloric Restriction on Acidic and Neutral Brain Glycosphingolipids in Adult Hexb Mice**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Genotype</th>
<th>Sulfatides (mg/100 mg dry wt.)</th>
<th>Cerebrosides (mg/100 mg dry wt.)</th>
<th>GA2 (mg/100 mg dry wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>+/?</td>
<td>2.0 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>–</td>
</tr>
<tr>
<td>AL</td>
<td>–/–</td>
<td>1.5 ± 0.2*</td>
<td>1.3 ± 0.1**</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>CR</td>
<td>–/–</td>
<td>1.6 ± 0.1*</td>
<td>1.2 ± 0.1**</td>
<td>2.7 ± 0.2</td>
</tr>
</tbody>
</table>

*a* Determined from densitometric scanning of HPTLC as shown in Figures 3 and 4.

*b* Determined from HPTLC analysis as described in Materials and Methods.

**p < 0.05 vs. Hexb+/? mice (two-tailed t-test).**

**p < 0.01 vs. Hexb+/? mice (two-tailed t-test).**
CD68 and F4/80 was significantly higher in the brains of the Hexb−/− mice than in those of the Hexb+/+ mice. Moreover, CR reduced expression of these inflammatory-associated markers in the Hexb−/− mice, suggesting that CR targets the inflammatory phenotype of SD. In addition, the number of apoptotic cells was markedly higher in the brains of the Hexb−/− mice than in those of the Hexb+/+ mice, and CR noticeably reduced the number of apoptotic cells in the Hexb−/− mice (unpublished observations). Further studies will be necessary to determine the extent of the anti-inflammatory effects of CR in SD and in other ganglioside storage diseases.

Our findings in the mice with SD support our previous studies in mice with epilepsy and brain cancer showing that mild to moderate CR reduces blood glucose and elevates ketone bodies and that these changes are accurate biomarkers of CR (Greene et al., 2001; Seyfried et al., 2003; Mantis et al., 2004; Seyfried and Mukherjee, 2005). The mature brain derives almost all of its energy from the aerobic oxidation of glucose under normal physiological conditions but will oxidize ketone bodies (β-OHB and acetoacetate) for energy during fasting or CR (Owen et al., 1967; Clarke and Sokoloff, 1999; Greene et al., 2001; Mantis et al., 2004). The metabolism of ketone bodies for energy is bioenergetically more efficient than the metabolism of glucose and also reduces oxygen free radicals production, because the cytosolic free NAD+/NADH concentration couple is in near-equilibrium with the glutathione couple (Veech, 2004; Masuda et al., 2005). Oxygen free radicals contribute to tissue inflammation, so we suggest that the energy transition from glucose to ketone bodies contributes to the antiinflammatory and neuroprotective effects of CR. This energy transition might also elevate brain-derived neurotrophic factor (BDNF) and Sir2, which have neuroprotective and anti-inflammatory properties (Maswood et al., 2004; Cohen et al., 2004). We also do not exclude the possibility that pro-inflammatory components in the standard mouse chow might contribute to CNS inflammation in SD mice under AL feeding and that CR simply reduces these components. Further studies involving dietary modifications will be needed to distinguish potential anti-inflammatory mechanisms.

Previous studies showed that NSAIDs improved motor coordination and survival in the Hexb−/− mice (Jeyakumar et al., 2004). As with NSAIDs, CR similarly slowed the decline of disease progression during the symptomatic phase of SD. It is interesting to note that both CR and NSAIDs improved behavior without altering the primary disease phenotype, i.e., ganglioside accumulation and storage. In contrast to NSAID therapy, which can involve adverse effects, including stomach ulcers, bruising, fluid retention, kidney problems, headache, dizziness, drowsiness, mouth sores, and skin rashes, CR has no adverse effects (Akarcı, 2005; Titchen et al., 2005). Indeed, CR enhances physiological fitness and general health (Keenan et al., 1999; Greene et al., 2001). Considered together, these findings suggest that CR alone or in combination with low doses of NSAIDs might be a preferred treatment for addressing the inflammatory component of SD.

The CR-induced improvements in survival and behavior of SD mice were not associated with reductions in the total content of gangliosides or in the content of GM2 and GA2. Consistently with these findings, CR did not noticeably reduce cytoplasmic neuronal storage vacuoles or dystrophic neurons. The reductions in cerebrosides and sulfatides in the SD mice are consistent with previous findings of dysmyelination in human patients and in animal models of ganglioside storage disease (Sandhoff et al., 1971; Kaye et al., 1992). CR had no effect on the content or composition of brain cerebrosides or sulfatides. A previous study...
suggested that phospholipid abnormalities contribute to the neuropathophysiology in SD mice (Buccoliero et al., 2004). With the exception of a slight reduction in phosphatidylcholine content, no noticeable differences were found between control and SD mice for phospholipid distribution. In addition, CR had no noticeable effect on the distribution of most brain lipids. We do not exclude the possibility that CR might influence some brain lipid molecular species, but further studies using gas-liquid chromatography would be needed to support this possibility. Taken together our findings indicate that CR improves behavior and extends longevity without producing marked changes in glycosphingolipid metabolism or in brain lipid distribution.

In summary, our results show that CR is effective in improving motor coordination and extending longevity in Hexb−/− mice during the symptomatic phase of SD, when extensive inflammatory processes are occurring. Furthermore, this improvement was observed without changes in brain glycosphingolipid composition or cytoplasmic neuronal vacuoles and was not associated with observable adverse effects. We suggest that the most effective therapeutic strategy for the life-long management of SD and other ganglioside storage diseases should involve combinatorial therapies, to include SRT (iminosugars, i.e., NB-DGJ and NB-DNJ), ERT (neural stem cells), and anti-inflammatory approaches (CR and NSAIDs).
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REFERENCES


