Supplementary Materials and Methods

Animal tissue collection

At 3 weeks of age, mice were deeply anesthetized with an intraperitoneal injection of xylazine (30 mg/kg)/ketamine (300 mg/kg) and when insensate, transcardially perfused with saline. The right cerebral and cerebellar hemispheres and a liver sample were removed and frozen at -80°C for later biochemical analyses. Mice were quickly re-perfused with 4% paraformaldehyde in 0.1M phosphate buffer (PB) and the remaining brain and liver tissue immersion fixed overnight in 4% paraformaldehyde in 0.1M PB. The following day, tissues were washed with PB and stored at 4°C for later staining.

Visualization of ganglioside and cholesterol accumulation by immunohistochemical and filipin labeling

Immunoperoxidase staining using mouse monoclonal IgM anti-GM2 ganglioside (1:200 dilution; clone 10-11 cell culture supernatant; produced in-house[1, 2]) and mouse monoclonal IgG anti-GM3 ganglioside (1:30 dilution; DH2 culture supernatant, 10-011; GlycoTech, Gaithersburg, MD) was carried out essentially as described.[3] Briefly, 35 μm thick coronal brain sections were quenched with H_2O_2, and then blocked and incubated with primary antibodies overnight at 4°C in the presence of 0.02% saponin for permeabilization. The following day after washing, sections were incubated with biotinylated secondary goat anti-mouse antibodies (1:200) with saponin, followed by standard processing with the Vectastain ABC kit (PK-4000) and the 3,3'-diaminobenzidine (DAB) Peroxidase Substrate Kit (SK-4100) from Vector Laboratories (Burlingame, CA). Sections were mounted and coverslipped using Permount (Sigma-Aldrich SP15-500).

Combined immunofluorescence/filipin labeling was carried out as previously described.[3] Liver vibratome sections were stained with rat monoclonal IgG anti-CD68 (1:200 dilution; FA-11, MCA1957; AbD Serotec, Raleigh, NC), followed by Alexa Fluor 488 goat anti-rat IgG (1:300 dilution; A11006 Invitrogen/Molecular Probes) both in the presence of 0.02% saponin. Following washing, sections were incubated (room temperature, 20 min.) with 0.005% (w/v) filipin complex [from *Streptomyces filipinensis* (Sigma-Aldrich F9765, St. Louis, MO)] in PBS, prepared from a 25mg/ml filipin stock dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich D2650). Sections were coverslipped with Prolong Gold Antifade mounting medium (P36934; ThermoFisher Scientific/Molecular Probes). Filipin labeling was also carried out alone on liver or brain with the same incubation conditions above. Negative controls included primary antibody omission, substitution with equivalent non-immune immunoglobulin fraction, and/or use of equivalent DMSO dilution lacking filipin.

Auditory brainstem responses

ABRs were used as an electrophysiological index of hearing thresholds and were assessed 18-24 hours after first CD injection at 8 weeks of age and subsequently at 12 and ~28 weeks. Mice were anesthetized with xylazine (4.5 mg/kg)/ketamine (90 mg/kg) and ABRs were elicited by clicks (100 microsecond duration) presented monaurally via a narrow plastic tube inserted into the left ear canal at seven different sound pressure levels (SPLs) in separate stimulation blocks. SPLs were measured via a sound level meter (Bruel and Kjaer, type 2236) with its microphone placed at the opening of the plastic tube. Clicks were presented at a rate of 5/sec. ABRs were recorded via subcutaneous needle electrodes placed at the vertex (active),

ventrolateral to the left ear (reference), and at the back of the head (ground).[4] ABRs were recorded using a BIOPAC EEG acquisition system (MP100) and AcqKnowledge software (BIOPAC Systems, Inc.) at a sample rate of 40 kHz and a gain of 50,000. EEG data were bandpass-filtered from 20 Hz to 3 kHz. ABR waveforms were obtained at each SPL by averaging responses to 150 stimulus presentations. ABR recordings and determination of hearing thresholds were performed by two experienced individuals blind with respect to treatment group. Threshold was defined as the minimum SPL at which at least one component of the ABR was detectable above baseline.[4] If ambiguous, recordings were repeated to verify presence of a genuine waveform component.

Differences in mean hearing threshold across treatment groups were statistically evaluated via planned unpaired non-directional *t*-tests (alpha level $p = 0.05$). For these analyses, thresholds at 34 dB and above 100 dB SPL (no discernable ABR components at 100 dB SPL) were nominally assigned values of 40 and 100 dB SPL, respectively. As there were no clear differences in thresholds between male and female mice, statistical analyses were performed on data collapsed across sex.

Determination of substrate solubilization by CDs

The following lipid substrates were tested for complexation with CDs: cholesterol (C3045, Sigma Aldrich, St. Louis, MO), GM2 ganglioside (G8397, Sigma Aldrich), GM3 ganglioside (860058-P, Avanti Polar Lipids, Inc., Alabaster, AL), 24(*S*)-hydroxycholesterol (BML-GR230, Enzo Life Sciences, Farmingdale, NY), 27-hydroxycholesterol (11160-3, Research Plus, Inc., Barnegat, NJ), lactosylceramide (1507, Matreya LLC, Pleasant Gap, PA), glucosylceramide (1521, Matreya LLC), D-erythro-sphingosine (860490P, Avanti Polar Lipids, Inc.), Oleoyl BMP (857133P, Avanti Polar Lipids, Inc.), and oleic acid (O1008, Sigma-Aldrich). Biochemical determinations of substrate complexation by CDs were performed in at least one of three ways for each substrate, the first two based on a mobility shift caused by the complexing CD, and the third employing a traditional phase solubility isotherm technique. In the first, the capacity of each CD to interact with different substrates was tested at three of the following CD concentrations: 1, 5, 10, 50, 100, or 250 mg/mL deionised water. Amount of lipid substrates for the interaction (3-20 mg) were chosen based on their intrinsic solubility and analytical detection limit and compounds were incubated together in 1 mL of deionised water. Experimental controls consisted of substrate incubated without CD. All samples were incubated (24 hours, 25^{o}C) with a stir rate of 120 rpm, except for UC at 300 rpm. Samples were prepared for HPLC evaluation by filtering through a Macherey Nagel membrane or Millipore filter, pore size 0.45 μm. HPLC was carried out using a diode array UV detection system (Agilent, Type 1200) set at 210 nm. A binding constant (K) was calculated for each CD:substrate combination from retention time shift of the lipid substrate as a function of applied CD concentration.[5] Higher shifts in retention time indicate greater stability of the lipid:CD complex.

The second method, used for GM2 and GM3 gangliosides which showed substantial water solubility, was a capillary electrophoresis mobility assay. These experiments were performed in triplicate and conducted in 100 mM borate buffer as a background electrolyte (pH between 9.4-10.0). Samples were hydrodynamically injected (200 mbar*s) into 25.0/33.5 cm (effective/total length) unfused silica capillaries and detection was performed at a wavelength of 195 nm. The applied voltage was set to +20 kV (for neutral CD derivatives) or +10 kV (for charged derivatives) to compensate for Joule-heating. For preconditioning, the capillary was rinsed with water, 0.1N sodium hydroxide, and water for 1 minute each and then with the running buffer for

2.5 minutes. Data were evaluated with the x-reciprocal method at three concentration levels (5, 10, and 15 mM) for each CD derivative,[6, 7] and an apparent average K, corresponding to the negative of the slope of mobility shift/[CD] vs. mobility shift was calculated.

The above techniques were used where the test compound was prohibitively expensive, showed substantial water solubility (as for the gangliosides) and/or was difficult to detect by UV absorbance above 220nm, preventing the ability to conduct standard phase-solubility isotherm studies. Wherever possible the following methodology was also used. Excess amounts of substrate were equilibrated with CD solutions of different concentrations for 24 hours. The solubilized amount of substrate was determined after membrane filtration by HPLC, and Ks were also calculated where possible according to Higuchi-Connors.[8] The methodology employed for each substrate is indicated in Table 2.

References

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