Inhibition of calcium uptake via the sarco/endoplasmic reticulum

$\text{Ca}^{2+}$-ATPase (SERCA) in a mouse model of Sandhoff disease,

and prevention by treatment with N-butyldeoxynojirimycin

Dori Pelled$^{1,3}$, Emyr Lloyd-Evans$^{1,2,3}$, Christian Riebeling$^1$, Mylvaganam Jeyakumar$^2$, Frances M. Platt$^2$, and Anthony H. Futerman$^1$

$^1$Department of Biological Chemistry,
Weizmann Institute of Science,
Rehovot 76100,
Israel

and

$^2$Glycobiology Institute, Department of Biochemistry,
University of Oxford,
South Parks Road,
Oxford OX1 3QU,
UK

Correspondence should be addressed to A.H. Futerman at: Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel.
Fax; (972)-8-9344112; Tel; (972)-8-9342704: e-mail: tony.futerman@weizmann.ac.il

$^3$ Both authors contributed equally to this work
Running title: Inhibition of SERCA in Sandhoff disease
Summary

Gangliosides are found at high levels in neuronal tissues where they play a variety of important functions. In the gangliosidoses, gangliosides accumulate due to defective activity of the lysosomal proteins responsible for their degradation, usually resulting in a rapidly progressive neurodegenerative disease. However, the molecular mechanism(s) leading from ganglioside accumulation to neurodegeneration is not known. We now examine the effect of ganglioside GM2 accumulation in a mouse model of Sandhoff disease (one of the GM2 gangliosidoses), the Hexb-/- mouse. Microsomes from Hexb-/- mouse brain showed a significant reduction in the rate of Ca$^{2+}$-uptake via the sarco/endoplasmic reticulum Ca$^{2+}$-ATPase (SERCA), which was prevented by feeding Hexb-/- mice with N-butyldeoxynojirimycin (NB-DNJ), an inhibitor of glycolipid synthesis that reduces GM2 storage. Changes in SERCA activity were not due to transcriptional regulation, but rather to a decrease in $V_{\text{max}}$. Moreover, exogenously-added GM2 had a similar effect on SERCA activity. The functional significance of these findings was established by the enhanced sensitivity of neurons cultured from embryonic Hexb-/- mice to cell death induced by thapsigargin, a specific SERCA inhibitor, and by the enhanced sensitivity of Hexb-/- microsomes to calcium-induced calcium-release. This study suggests a mechanistic link between GM2 accumulation, reduced SERCA activity, and neuronal cell death, which may be of significance for delineating the neuropathophysiology of Sandhoff disease.
Introduction

The GM2 gangliosidoses are a group of inherited metabolic disorders caused by mutations in any of three genes, the HEXA gene, resulting in Tay Sachs disease, the HEXB gene, resulting in Sandhoff disease, and the GM2A gene, resulting in GM2 activator deficiency (1,2). The HEXA and HEXB genes code for the α-hexosaminidase α- and β-subunits respectively, which dimerize to produce two forms of the enzyme, A (ab) and B (bb), and a minor form, S (aa). In both Tay-Sachs and Sandhoff disease (α- and β-subunit deficiency, respectively), there is a deficit of hexosaminidase A (ab), and as a consequence, massive accumulation of ganglioside GM2 in the brain (3). As in all lysosomal storage diseases, significant clinical heterogeneity is observed, varying from infantile-onset, rapidly progressive neurological disease culminating in death before 4-years of age, to late-onset, sub-acute or chronic progressive neurological disease (4).

Few molecular details are available that delineate the pathway leading from GM2 accumulation to neurological disease. In the current study, we analyze the effect of GM2 accumulation in neuronal tissues obtained from mice homozygous for the disrupted HEXB gene (5,6), a model of Sandhoff disease. The Hexb−/− mouse shows increased neuronal GM2 storage in most brain areas (5), neuronal apoptosis (6), changes in rates of axonal and dendritic growth (7), and severe neurological disturbances causing death by ~4 months of age. We demonstrate that brain microsomes and neurons cultured from these mice show dramatically reduced levels of Ca\(^{2+}\)-uptake via the sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA\(^1\)), which is not related to transcriptional regulation of SERCA, but rather to changes in V\(_{\text{max}}\). Impairment of SERCA activity usually results in neuronal cell dysfunction and/or death (8,9), and our observations that Ca\(^{2+}\)-uptake via SERCA is severely impaired upon GM2 accumulation may suggest a molecular mechanism to explain, at least in part, the neuropathophysiology in Sandhoff disease. This is supported by our observation that
SERCA activity is essentially normal in 3-month-old Hexb-/- mice fed with the glycolipid synthesis inhibitor N-butyldeoxyoijirimycin (NB-DNJ) (10,11), which correlates with the delayed symptom onset and increased life expectancy of these mice (12).
Experimental procedures

Materials

Gangliosides GM3, GM2 and GM1 were obtained from Matreya (Pleasant Gap, PA, USA). Antipyrilazo III, thapsigargin, creatine phosphokinase, phosphocreatine, and ATP, were from Sigma. $^{45}\text{Ca}^{2+}$ (30 mCi/mg) was from Amersham Pharmacia Biotech, UK. N-butyldeoxynojirimycin (NB-DNJ) was from Searle Monsanto and Oxford GlycoSciences. The anti-SERCA2 antibody (N-19) was from Santa Cruz Biotechnology (Santa Cruz, CA), and a horseradish peroxidase-conjugated rabbit anti-goat secondary antibody was from Jackson Laboratories (West Grove, PA). Silica gel 60 thin layer chromatography plates were from Merck (Darmstadt, Germany). All solvents were of analytical grade and were purchased from Biolab (Jerusalem, Israel). Oligonucleotides were synthesized by the Weizmann Institute Oligonucleotide and Peptide Synthesis facility.

Hexb colony

A mouse model of Sandhoff disease (the Hexb−/− mouse) (5-7) was maintained in the Experimental Animal Center of the Weizmann Institute of Science (7). Wild type (Hexb+/+) mice were bred with each other, as were Hexb−/− mice, to obtain homozygous offspring. The genotype of the mice was determined by the polymerase chain reaction (PCR) using genomic DNA extracted from mouse tails (13). A similar Hexb colony, maintained in the Glycobiology Institute at the University of Oxford, UK, was used for the studies in which Hexb−/− mice were fed with NB-DNJ. Mice were treated from weaning at 3 weeks with 1200 mg/kg/day of NB-DNJ.

Lipid analysis

GM2 levels were determined in Hexb−/− mouse brains at various ages after birth and compared to Hexb+/+ mice (11). GM2 levels were determined in microsomes after glycosphingolipid extraction (14,15), separation by thin layer chromatography using
chloroform:methanol:9.8mM CaCl\(_2\) (60:35:8, vol:vol:vol) as the developing solvent, and resorcinol staining. Ganglioside GM2 was identified using an authentic standard.

**Hexb brain microsomes**

Hexb mice were sacrificed after 3-4 months, their brains removed, separated into cerebral cortex and cerebellum, rapidly frozen in liquid N\(_2\), and stored at -80°C; in one set of experiments, microsomes were prepared from embryonic day 17 (E17) mouse cortices. Cortical microsomes (from 12-25 mice) were prepared essentially as described (16) with some modifications. Tissue was suspended at a ratio of 1:4 (wt/vol) in ice cold 0.32 M sucrose, 20 mM HEPES-KOH, pH 7.0, containing 0.4 mM phenylmethylsulfonylfluoride, leupeptin (0.8 µg/ml) and aprotinin (1.4 TIU) (buffer A), and homogenized at 4°C using 8 up and down strokes of a rotating Potter-Elvehjem homogenizer. After centrifugation (700 g\(_{av}\), 10 min), the resulting pellet (P1) was gently resuspended in 1/4 of the original volume of buffer A, centrifuged (700 g\(_{av}\), 10 min), and the two supernatants pooled (S1). Mitochondria were removed by centrifugation (8,000 g\(_{av}\), 45 min) of S1 and the resulting supernatant (S2) centrifuged (115,000 g\(_{av}\), 90 min) to obtain a microsomal pellet (P3), which was resuspended in 0.4-0.8 ml of buffer A. Protein was determined (17) and the microsomes subsequently flash-frozen in liquid N\(_2\). Microsomes were stored at -80°C and used for up to several weeks after their preparation, during which time there was no change in their activity with respect to Ca\(^{2+}\)-release and uptake.

**Spectrophotometric assay of Ca\(^{2+}\)-uptake**

Ca\(^{2+}\)-uptake was measured by a spectrophotometric assay using the Ca\(^{2+}\)-sensitive dye, antipyrylazo III (18-20), with some modifications. Mouse brain microsomes (330 µg in 8-15 µl buffer A) were added to 0.95 ml of 8 mM NaMOPS, pH 7.0, 40 mM KCl, 62.5 mM K\(_2\)HPO\(_4\), and 250 µM antipyrylazo III, in a plastic cuvette containing a magnetic stir bar, to which 1 mM MgATP,
40 µg/ml creatine phosphokinase and 5 mM phosphocreatine, pH 7.0, were added. Ca\(^{2+}\)-uptake and release were measured in a Cary spectrophotometer (Varian Australia Pty Ltd.) at 37°C by subtracting A\(_{790}\) from A\(_{710}\) at 2 second intervals. The effect of exogenously-added GM3, GM2 and GM1, dissolved in absolute ethanol, was tested by their addition 5 min before Ca\(^{2+}\)-loading. The rate of Ca\(^{2+}\)-uptake into microsomes was calculated by measuring the linear portion of the slope after addition of Ca\(^{2+}\).

**Kinetic assay of SERCA**

Ca\(^{2+}\)-uptake by SERCA was determined radiometrically using a rapid filtration technique (21). Mouse cortical microsomes (350 µg protein) were incubated at 37°C in 1.5 ml of buffer B (40 mM imidazole, pH 7.0, 100 mM KCl, 5 mM MgCl\(_2\), 5 mM NaN\(_3\), 5 mM potassium oxalate, 0.5 mM EGTA, 1 µM ruthenium red (21) (which blocks spontaneous Ca\(^{2+}\)-release via the ryanodine receptor (22)), 10 µCi \(^{45}\)Ca\(^{2+}\) and CaCl\(_2\), to yield the required final concentration of free Ca\(^{2+}\) (determined using an algorithm (23) and software available at [http://www.stanford.edu/~cpatton/maxc.html](http://www.stanford.edu/~cpatton/maxc.html)).

Ca\(^{2+}\)-uptake was initiated by addition of ATP, and terminated after 1, 3 and 5 sec by addition of 3 ml of ice-cold washing solution (20 mM HEPES, pH 7.4, 150 mM KCl, 1.4 mM MgCl\(_2\), and 2 mM KH\(_2\)PO\(_4\)) (24), followed by filtration through a HAWP 0.45 µm Millipore filter in a Millipore filtration device. The initial rate of Ca\(^{2+}\)-uptake (v\(_o\)) was calculated by linear regression analysis. The kinetic coefficients of Ca\(^{2+}\)-uptake were calculated according to (25),

\[
    v = V_{\text{max}} \frac{K_{Ca}^n}{[Ca_i]^n + K_{Ca}^n}
\]

(eqn. 1), where \(V_{\text{max}}\) is maximum velocity, \(K_{Ca}\) is the concentration required for half-maximal activation, and \(n\) is the equivalent of the Hill coefficient), using non-linear regression analysis (Origin 5.0, MicroCal Software, Inc.). The K\(_{\text{ATP}}\) was obtained by Michaelis-Menten analysis.
Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was prepared from individual cortices using the TRI reagent (MRC, Cincinnati, OH) or from ~0.5 x 10^6 neurons (see below) grown on 24 mm cover slips using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturers' instructions. The reverse transcription-polymerase chain reaction (RT-PCR) was performed as described (26) using the QIAGEN OneStep RT-PCR kit (Qiagen, Hilden, Germany). Primers (SERCA2a: 5'-ACTTCTTGATCCTCTACGTG and 5'-AAATGGTTTAGGAAGCGGTT, 33 cycles, annealing temperature 53°C; SERCA2b: 5'-ACTTCTTGATCCTCTACGTG (same as SERCA2a) and 5'-AGACCAGAACATATCGCTAA, 25 cycles, annealing temperature 53°C; glyceraldehyde-3-phosphate dehydrogenase: 5'-TTAGCACCCCTGGCCAAGG and 5'- CTTACTCCTTGGAGGCCATG, 23 cycles, annealing temperature 50°C) were designed using the MacMolly Tetra program (Softgene, Berlin, Germany) and conditions were adjusted to be within the linear phase of PCR. For sequencing, bands were excised from gels and purified using the QIAquick gel extraction kit (QIAGEN, Hilden, Germany) and subsequently sequenced by the DNA sequencing unit of the Weizmann Institute of Science using the respective primers.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting was performed as described (27) using a 6% separating gel. After transfer to nitrocellulose membranes, the blot was incubated with blocking buffer (Tris-buffered saline containing 3% (w/v) non-fat dried milk, 1% (w/v) bovine serum albumin and 0.1% Tween 20) for 20 min and then incubated with an anti-SERCA2 antibody at a dilution of 1/100 in blocking buffer for 2 h. Bound antibodies were detected after washing using 25 ng/ml horseradish peroxidase-conjugated rabbit
anti-goat secondary antibodies in blocking buffer for 1 h and the SuperSignal chemiluminescent
detection reagent (Pierce, Rockford, IL).

**Neuronal Ca\(^{2+}\)-uptake**

Embryos (embryonic day 17) were isolated, the hippocampi removed and hippocampal
pyramidal neurons cultured as described (7). The dissected hippocampi were dissociated by
trypsinization (0.25% w/v, for 15 min at 37\(^{\circ}\)C), the tissue washed in Mg\(^{2+}\)/Ca\(^{2+}\)-free Hank's
balanced salt solution, and dissociated by repeated passage through a constricted Pasteur pipette.
Neurons were maintained in Neurobasal serum-free medium containing B27 supplements and L-
glutamine (28) (Life Technologies Inc., Paisley, UK).

For analysis of Ca\(^{2+}\)-uptake (29), neurons were plated at a density of 1.1 \times 10^6 cells per 60
mm culture dish that contained three 24 mm polylysine-coated coverslips. Coverslips were washed
in Ca\(^{2+}\)-free medium (minimal essential medium containing 50 mM HEPES (pH 7.3), 4 mM
NaHCO\(_3\), 11 mg/ml pyruvic acid, 1 mM glutamine and 0.6% (w/v) glucose (30)), and transferred to
a new dish containing the same medium. After 25 min at 37\(^{\circ}\)C, neurons were incubated with the
calcium ionophore A23187 (1 µM) (31) for 5 min, prior to addition of 1 µCi \(^{45}\)Ca\(^{2+}\) for 30 sec at
37\(^{\circ}\)C (32). The reaction was terminated by removing coverslips from the wells, washed by dipping
5-times in Ca\(^{2+}\)-free medium, and then placing in 0.65 ml NaOH (0.5 M) for 3 h. \(^{45}\)Ca\(^{2+}\) was further
extracted by adding NaOH for 16 h, and then for another 2 h. NaOH extracts were pooled and
\(^{45}\)Ca\(^{2+}\) determined by liquid scintillation counting.

**Neuronal cell death**

Neurons were plated at a density of 25,000 cells per 13 mm cover slip in a 24-multiwell dish.
Live and dead cells were distinguished using 2 µM calcein acetoxymethyl ester and 4 µM ethidium
homodimer-1, respectively, as described in a Live/Dead\(^{\circledR}\) viability/cytotoxicity kit (Molecular Probes
Inc., OR). At least 700-800 cells were counted per coverslip. Neurons were examined using a Plan 25x/0.45 numerical aperture objective of a Zeiss Axiovert 35 microscope.
Results

Previous studies have shown that GM2 accumulates in adult Hexb-/- mouse brains (5). We now examine GM2 accumulation throughout brain development. Even at the earliest age measured, embryonic day 10, small but significant levels of GM2 were detected (7.6 ± 0.4 µg/10 mg dry weight), which increased to levels of 162 ± 8 µg/10 mg dry weight in 3-month-old mice (Fig. 1A), whereas no GM2 could be detected at any stage in Hexb+/+ brains. Significant amounts of GM2 were also detected in microsomes prepared from 3-4 month-old Hexb-/- mice, but essentially no GM2 was detected in microsomes from Hexb+/+ mice (Fig. 1B). A significant decrease in GM2 accumulation (Fig. 1B) was observed in microsomes from 120-day-old Hexb-/- mice that had been fed with NB-DNJ, similar to that previously reported for whole brain (12).

We recently proposed that changes in Ca\textsuperscript{2+}-homeostasis, and specifically changes in Ca\textsuperscript{2+}-release from intracellular stores via the ryanodine receptor (29,33), may be responsible, at least in part, for the neuropathophysiology observed in neuronopathic forms of Gaucher disease, in which glucosylceramide accumulates in lysosomes. To determine if Ca\textsuperscript{2+}-homeostasis is also affected in Sandhoff disease mice, we examined Ca\textsuperscript{2+}-release and uptake in Hexb microsomes, by a spectrophotometric assay using the Ca\textsuperscript{2+}-sensitive dye, antipyrylazo III. No differences were detected in the rate of agonist-induced Ca\textsuperscript{2+}-release via the ryanodine or inositol 1,4,5-trisphosphate receptors, the major Ca\textsuperscript{2+}-release channels in the endoplasmic reticulum (ER) (not shown). In contrast, the rate of Ca\textsuperscript{2+}-uptake was reduced by ~5-fold in microsomes from Hexb-/- compared to Hexb+/+ mice (Fig. 2A), and remarkably, was almost completely normal in microsomes from Hexb-/- mice fed with NB-DNJ (Fig. 2B). These results suggest a causal relationship between GM2 levels and rates of Ca\textsuperscript{2+}-uptake via SERCA. Although we cannot formally exclude the possibility that NB-DNJ by itself effects SERCA activity in vivo, there is no indication from clinical studies
(34) or from studies in cell culture (35) that NB-DNJ has any effect on Ca\(^{2+}\)-homeostasis, and moreover, no changes are observed in GM2 levels in wild type mice fed NB-DNJ (36). A causal relationship between GM2 levels and SERCA activity was supported by a similar reduction in the rate of Ca\(^{2+}\)-uptake in Hexb\(^{++}\) microsomes incubated with exogenously-added GM2 (Table 1). Two other monosialogangliosides, GM3 and GM1, also reduced the rate of Ca\(^{2+}\)-uptake, but to a smaller extent than GM2 (Table 1).

To determine the mechanism by which GM2 affects SERCA activity, we analyzed the initial rate of Ca\(^{2+}\)-uptake by a radiometric assay using a rapid filtration technique (21). The \(V_{\text{max}}\) of SERCA was 3.70 ± 0.12 nmol Ca\(^{2+}\)/sec/mg of protein in Hexb\(^{++}\) brain microsomes and 1.88 ± 0.17 nmol Ca\(^{2+}\)/sec/mg of protein in Hexb\(^{-/-}\) microsomes, the \(K_{Ca}\) was 0.29 ± 0.03 µM and 0.23 ± 0.06 µM for Hexb\(^{++}\) and Hexb\(^{-/-}\), respectively, with no change in the Hill coefficient (1.9 vs. 2.0) (Fig. 3A), and there was a small but non-significant reduction in the \(K_{ATP}\) (140 ± 40 µM for Hexb\(^{++}\) and 80 ± 20 µM for Hexb\(^{-/-}\)) (Fig. 3B). Thus, GM2 affects the \(V_{\text{max}}\) of SERCA but not its affinity towards Ca\(^{2+}\) or ATP. The reduction in \(V_{\text{max}}\) was not due to transcriptional regulation of SERCA since there were no changes in mRNA expression in brain for the SERCA2 isoforms\(^2\) (the predominant isoform in brain (37)) (Fig. 4A), and no change in SERCA2 protein levels in microsomes (Fig. 4B).

We next examined the functional significance of the reduced rate of Ca\(^{2+}\)-uptake via SERCA. Hexb\(^{-/-}\) microsomes were significantly more sensitive to calcium-induced calcium release (CICR) than Hexb\(^{++}\) microsomes or microsomes from Hexb\(^{-/-}\) mice fed with NB-DNJ (Fig. 5A, B), suggesting that elevated cytosolic Ca\(^{2+}\) levels in Hexb\(^{-/-}\) neurons may induce CICR. In addition, thapsigargin (75µM), a specific SERCA inhibitor (38), blocked Ca\(^{2+}\)-uptake by only 50% in Hexb\(^{++}\) microsomes (compare Fig. 2B to Fig. 5C), but completely blocked SERCA activity in
Hexb-/- microsomes (Fig. 5C, D), an effect that was again prevented in Hexb-/- microsomes from NB-DNJ-fed mice. To determine whether the enhanced sensitivity to thapsigargin might be of functional significance for neuronal viability, we examined the effect of thapsigargin on neurons cultured from E17 Hexb mice. No changes in mRNA expression of SERCA2 was detected in hippocampal neurons (Fig. 4A). Even in neurons from these young mice, a significant reduction in the rate of Ca\(^{2+}\)-uptake into the ER of live neurons was observed (Fig. 6A) and the rate of Ca\(^{2+}\)-uptake into E17 microsomes was also decreased by ~50% (Fig. 6B). Note that the specific activity of SERCA in embryonic microsomes was ~50-fold lower than in adult microsomes (compare Figs. 2B and 6B), implying that the small but significant amounts of GM2 that accumulates in E17 brain (Fig. 1), and in cultured neurons from E17 embryos (7), is sufficient to account for the reduction in SERCA activity. Intriguingly, neurons cultured from Hexb-/- mice were more sensitive to thapsigargin-induced neuronal cell death than Hexb+/+ neurons, supporting our contention that changes in cytosolic Ca\(^{2+}\) levels resulting from inhibition of SERCA activity by GM2 may be involved in the molecular mechanism(s) causing neuronal pathophysiology in Sandhoff disease.
Discussion

The major finding of the current study is that ganglioside GM2, upon its accumulation in neuronal tissues from Hexb-/- mice, affects the rate of Ca\textsuperscript{2+}-uptake into brain microsomes and into the ER of live neurons, due to a decrease in the V_{max} of SERCA. As a result, neurons are more sensitive to thapsigargin-induced neuronal cell death, implying that altered Ca\textsuperscript{2+}-uptake may be of physiological relevance for understanding the etiology of Sandhoff disease.

The main functions of SERCA in skeletal and smooth muscle (37) are to prevent prolonged elevation of cytosolic Ca\textsuperscript{2+} and to maintain Ca\textsuperscript{2+} levels within the ER (39). Modification of SERCA activity normally has deleterious effects (40). Less is known about the regulation of SERCA activity in brain, where it occurs at much lower levels than in muscle (41), but a number of studies, mainly using thapsigargin, have shown that SERCA plays a crucial role in neuronal function. For instance, exposure of neurons and neuronal cell lines to thapsigargin results in ER stress (42,43), decreased neuronal viability (8,9), inhibition of protein synthesis (44), and injury in developing nerves (45).

Two previous studies have demonstrated that exogenously-added gangliosides modulate SERCA activity in rabbit sarcoplasmic reticulum (46,47), but ours is the first to show a direct physiological link between endogenous ganglioside accumulation and neuronal cell death mediated via SERCA. In the previous studies, GM1 inhibited rabbit sarcoplasmic reticulum SERCA whereas GM3 activated SERCA (47), via a mechanism that was proposed to involve the compactness of the hydrophilic and hydrophobic domains. In our study on mouse brain SERCA, GM1 and GM3 both inhibited SERCA, although exogenously-added GM3 had a slight stimulatory effect at higher concentrations (i.e. 50 µM) (not shown). Irrespective of the effects of GM3 and GM1, our data unambiguously demonstrate that GM2 has a potent inhibitory effect on SERCA activity, and that
neurons which accumulate endogenous GM2 are more sensitive to thapsigargin-induced neuronal cell death, via a pathway that may be amplified in neurons by their enhanced sensitivity to CICR.

We previously demonstrated that endogenous GM2 accumulation in Hexb-/- neurons results in reduced rates of axonal and dendritic growth, but no changes in viability under non-stress conditions (7). Interestingly, an inverse relationship exists between cytosolic $\text{Ca}^{2+}$ levels and rates of axon outgrowth (48), suggesting a mechanistic link between GM2 accumulation, SERCA activity, cytosolic $\text{Ca}^{2+}$, and axonal outgrowth. Clearly, modulation of SERCA activity, either directly or indirectly by GM2, is the critical step in this pathway, and a crucial question concerns whether the intracellular GM2 that accumulates in Hexb-/- brains is accessible to SERCA in neurons since gangliosides are not normally considered to reside in the ER, as they are synthesized distal to the ER in the Golgi apparatus. Our observation that GM2 is readily detectable in the same microsomes used to analyze SERCA activity lends support to the possibility that SERCA could be directly modulated by GM2. It should be noted that other gangliosides affect intracellular organelles other than those in which they reside or are degraded. For instance, ganglioside GD3 traffics to mitochondria where it affects mitochondrial function (49).

To date, no molecular mechanism has been provided to explain the neuropathophysiology in Sandhoff disease. Microglial activation precedes acute neurodegeneration in Hexb-/- mice, with elevation of various genes related to a macrophage-mediated inflammatory response (50,51), although the initiating signal for microglial activation is not known. Our data suggest that a downstream response to changes in cytosolic $\text{Ca}^{2+}$ levels might initiate a stress response (52), which may subsequently act as an initiating signal for the neuroinflammatory response. Interestingly, the inflammatory response pre-dates symptom onset in Hexb-/- mice (53), and changes in SERCA activity can be detected in mice as young as embryonic day 17, even though no symptoms of
Sandhoff disease are observed until 2-3 months of age. Moreover, the ability of NB-DNJ to prevent the reduction of SERCA activity, due to a decrease in storage levels of GM2, together with the delayed symptom onset of these mice (12), implies a direct correlation between the initiation of neuronal cell dysfunction and/or death and modulation of SERCA activity by GM2.
Acknowledgments

Hexb mice were obtained from the laboratory of Prof. Konrad Sandhoff and Dr. Gerhild van Echten-Deckert, University of Bonn, in the framework of a grant from the German-Israel Foundation for Scientific Research. Christian Riebeling is supported by a Research Training Network fellowship from the European Union (HPRN-CT-2000-00077), Mylvaganam Jeyakumar is supported by The Wellcome Trust, and Frances M. Platt is a Lister Institute Research Fellow. We thank Niv Tutka from the Experimental Animal Center (Weizmann Institute of Science) for maintaining the Hexb mouse colony, Revital Benvenisti and Omer Peretz for genotyping the Hexb colony, and Prof. Steve Karlish (Weizmann Institute of Science) for helpful comments.

Footnotes

1 Abbreviations used:
CICR, calcium-induced calcium-release;
ER, endoplasmic reticulum;
NB-DNJ, N-butyldeoxynojirimycin;
RT-PCR, reverse transcription-polymerase chain reaction;
SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis;
SERCA, sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase.

2 Using the primers for SERCA2a, a second product was amplified (Fig. 4A). Sequencing revealed a novel SERCA2 isoform with an alternative COOH-terminus, designated SERCA2c. This isoform differs from SERCA2a by 83 nucleotides (6879 to 6961, AJ131870 (54)) inserted between exons 21 and 25. The amino acid sequence of the COOH-terminus is LEQPGQS instead of 518-LEQPAILE for SERCA2a).
References


Table 1. Effect of exogenously-added gangliosides on microsomal Ca\(^{2+}\)-uptake. Cortical microsomes were incubated with gangliosides 5 min prior to Ca\(^{2+}\)-loading, as in Fig. 2. Results are means ± s.d. for 2-4 independent analyses.

<table>
<thead>
<tr>
<th>Ganglioside (10 µM)</th>
<th>Rate of Ca(^{2+})-uptake (nmol/sec/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.22 ± 0.01</td>
</tr>
<tr>
<td>GM3</td>
<td>0.17 ± 0.04</td>
</tr>
<tr>
<td>GM2</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>GM1</td>
<td>0.13 ± 0.00</td>
</tr>
</tbody>
</table>
Figure legends

**Fig. 1. GM2 accumulation in Hexb brains and microsomes.** A. Gangliosides were extracted from individual Hexb-/- mouse brains at the indicated ages and GM2 quantified. B. Gangliosides were extracted from cortical microsomes obtained from 120-day-old Hexb+/+ and Hexb-/- mice, or from Hexb-/- mice that were fed NB-DNJ, and ganglioside GM2 visualized by resorcinol.

**Fig. 2. Rate of Ca\(^{2+}\)-uptake in Hexb microsomes.** A. Cortical microsomes were loaded by two sequential additions of 25 nmol Ca\(^{2+}\). Data are representative traces showing absorbance change (A\(_{710}\)-A\(_{790}\)) of antipyrilazo III versus time, with an increase in absorbance demonstrating an increase in free Ca\(^{2+}\) in the cuvette, and a decrease in absorbance demonstrating a decrease in free Ca\(^{2+}\) due to microsomal Ca\(^{2+}\)-uptake by SERCA. B. The mean rate of Ca\(^{2+}\)-uptake (n=3 or 4, ± s.d.) was determined in microsomes obtained from Hexb+/+ and Hexb-/- mice, or from Hexb-/- mice that were fed NB-DNJ.

**Fig. 3. Initial rate of Ca\(^{2+}\)-uptake in Hexb microsomes.** The initial rate (\(v_i\)) of Ca\(^{2+}\)-uptake in cortical microsomes from Hexb+/+ (circles) or Hexb-/- (triangles) mouse brains was measured using \(^{45}\)Ca\(^{2+}\) varying either (A) free Ca\(^{2+}\) (in the presence of 5 mM ATP) or (B) ATP (in the presence of 10 µM free Ca\(^{2+}\)). A best fit was obtained by non-linear regression analysis. Results are means ± s.d. for 5-12 independent measurements using 2-3 different microsomal preparations.

**Fig. 4. RT-PCR and Western blotting of SERCA.** A. RT-PCR was performed on RNA extracted from two samples of Hexb+/+ and Hexb-/- cortices or from two cultures of hippocampal neurons; levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression are shown to demonstrate that equal amounts of RNA were used for RT-PCR. One of two independent experiments is shown. B. Western blotting using an anti-SERCA antibody was performed on three different microsomal preparations from each Hexb-/- and Hexb+/+ brain (100 µg of protein), or
from rat heart (20 µg of protein); note that even though SERCA2 is the predominant SERCA isoform in brain, its levels are much lower than in heart microsomes.

**Fig. 5. CICR and thapsigargin sensitivity.** (A,B) Cortical microsomes were loaded by sequential additions of 25 nmol Ca\(^{2+}\) until CICR was induced. Panel A gives the average concentration of Ca\(^{2+}\) required to induce CICR for microsomes from Hexb\(^{+/+}\) and Hexb\(^{-/-}\) microsomes, or from microsomes from Hexb\(^{-/-}\) mice that were fed NB-DNJ, and panel B shows typical traces of absorbance change (\(A_{710}\) - \(A_{790}\)) of antipyrylazo III versus time; in the upper trace, CICR is induced after 5 additions of 25 nmol Ca\(^{2+}\), whereas in the bottom trace, CICR is induced after 3 additions of 25 nmol Ca\(^{2+}\). (C, D) Cortical microsomes were incubated with 75 µM thapsigargin (not shown), followed by one addition of 25 nmol Ca\(^{2+}\). Panel C gives the rate of Ca\(^{2+}\)-uptake after thapsigargin addition, and panel D shows typical traces of Ca\(^{2+}\)-uptake. Results in panels A and C are means ± s.d. of 3-5 independent experiments.

**Fig. 6. Ca\(^{2+}\)-uptake in embryonic hippocampal neurons.** A. \(^{45}\)Ca\(^{2+}\)-uptake into 7-day-old neurons cultured from E17 Hexb\(^{+/+}\) and Hexb\(^{-/-}\) mice. Results are means ± s.d. from 3 independent neuronal cultures in which \(^{45}\)Ca\(^{2+}\)-uptake was measured on 6 individual coverslips. B. The mean rate of Ca\(^{2+}\)-uptake (n=3 or 4, ± s.d.) was determined in cortical microsomes from E17 Hexb\(^{+/+}\) and Hexb\(^{-/-}\) mice. Results are means ± s.d. for 3 independent measurements. C. 4-day-old neurons cultured from E17 Hexb\(^{+/+}\) (squares) or Hexb\(^{-/-}\) (circles) mice were incubated with thapsigargin for 1 hour and cell death quantified using the Live/Dead kit. Results are means ± s.d. for three independent cultures in which 4 coverslips were analyzed for each thapsigargin concentration.
Fig. 1

A

GM2 (µg/10 mg dry wt)

Age (days)

Hexb+/+  Hexb/-  Hexb/- + NB-DNJ

GM2

B
Fig. 2

A

Hexb+/+

Hexb-/-

B

Rate of Ca$^{2+}$-uptake (nmol/sec/mg protein)

+/

-/

-/

+ NB-DNJ

Fig. 2
Ca\textsuperscript{2+} uptake (nmol/sec/mg protein)

Free calcium [M]

ATP [mM]

Fig. 3
Fig. 4
Fig. 5

A. Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+}-release (nmol Ca\textsuperscript{2+}-added)

B. Graph showing Ca\textsuperscript{2+} release in different conditions.

C. Rate of Ca\textsuperscript{2+}-uptake (nmol/sec/mg protein)

D. Graph showing Ca\textsuperscript{2+} uptake in different conditions.
Fig. 6

A

$\text{Ca}^{2+}$ uptake (nmol/sec/mg protein)

B

Rate of $\text{Ca}^{2+}$ uptake (nmol/sec/mg protein)

C

Dead cells (percent)

Thapsigargin [$\mu$M]
Inhibition of calcium uptake via the sarco/endoplasmic reticulum Ca2+-ATPase (SERCA) in a mouse model of Sandhoff disease, and prevention by treatment with N-butyldeoxynojirimycin
Dori Pelled, Emyr Lloyd-Evans, Christian Riebeling, Mylvaganam Jeyakumar, Frances M. Platt and Anthony H. Futerman

J. Biol. Chem. published online May 19, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M302964200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts