Alleviation of neuronal ganglioside storage does not improve the clinical course of the Niemann–Pick C disease mouse

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Niemann–Pick disease Type C (NP-C) is a progressive neurodegenerative disorder caused by mutations in the NPC1 gene and characterized by intracellular accumulation of cholesterol and sphingolipids. The major neuronal storage material in NP-C consists of gangliosides and other glycolipids, raising the possibility that the accumulation of these lipids may participate in the neurodegenerative process. To determine if ganglioside accumulation is a crucial factor in neuropathogenesis, we bred NP-C model mice with mice carrying a targeted mutation in GalNAcT, the gene encoding the β-1-4GalNAc transferase responsible for the synthesis of GM2 and complex gangliosides. Unlike the NP-C model mice, these double mutant mice did not exhibit central nervous system (CNS) accumulation of gangliosides GM2 or of glycolipids GA1 and GA2. Histological analysis revealed that the characteristic neuronal storage pathology of NP-C disease was substantially reduced in the double mutant mice. By contrast, visceral pathology was similar in the NP-C and double mutant mice. Most notably, the clinical phenotype of the double mutant mice, in the absence of CNS ganglioside accumulation and associated neuronal pathology, did not improve. The results demonstrate that complex ganglioside storage, while responsible for much of the neuronal pathology, does not significantly influence the clinical phenotype of the NP-C model.

INTRODUCTION

Niemann–Pick disease Type C (NP-C) is an inherited lipid storage disorder affecting both the visceral organs and the nervous system (1,2). The majority of patients have defects in the recently obtained NPC1 gene (3,4). Less than 5% of patients carry mutations in a gene other than NPC1. A wide spectrum of clinical phenotypes exists for NP-C. However, the dominant feature is severe and progressive neurodegeneration that ultimately results in a shortened life span.

Unlike other lipidoses, which are caused by mutations in genes encoding enzymes and activator proteins required for lysosomal degradation of lipid substrates, NP-C is caused by an error in intracellular trafficking. Emblematic of the cellular defect in NP-C is the impaired ability to redistribute endocytosed low density lipoprotein (LDL)-derived cholesterol (5,6). As a result, unesterified cholesterol accumulates in lysosomes and in the trans-Golgi apparatus. However, in NP-C patients, lipid storage is not restricted to just cholesterol (7). In the liver, other lipid metabolites in addition to cholesterol accumulate including sphingomyelin, bis(monoacylglycero) phosphate, glycosphingolipids and free sphingosine. In the central nervous system (CNS), which is severely affected, there is little detectable cholesterol accumulation and no phospholipid accumulation. Instead, glycosphingolipids—both neutral glycolipids and gangliosides—are the predominant storage material.

The prominent neuronal ganglioside storage in NP-C has been implicated as a possible factor causing CNS degeneration (8–11). To investigate directly the role of ganglioside storage in NP-C, we introduced a genetic lesion into NP-C mice that prevented synthesis of GM2 and more complex gangliosides (12). We found that the neuronal storage pathology characteristic of NP-C was substantially reduced in these double mutant mice. Surprisingly, even with the amelioration of pathology, the clinical manifestations of the double mutant did not improve, demonstrating that neuronal ganglioside storage is not a significant factor in the clinical phenotype of the NP-C model mice.

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RESULTS

Experimental design

A murine model of NP-C, with an inactivating mutation in the \(\text{NPC1}\) gene, shares genetic, pathological and clinical features with the acute form of the human disease (4,13–18). The NP-C mice display neuronal storage as a major pathological manifestation in their CNS. They exhibit severe neurological disturbances and have a shortened life span. To determine the consequence of complex ganglioside storage on the neuropathology and clinical phenotype of the NP-C mice, double mutant mice were established that were homozygous for both the inactivated \(\text{NPC1}\) gene and a disrupted \(\text{GalNAcT}\) gene (12). The \(\text{GalNAcT}\) gene encodes a \(\beta_1-4\) GalNAc transferase that is required for the synthesis of complex gangliosides (Fig. 1). \(\text{GalNAcT}^{-/-}\) single mutant mice have a normal life span and relatively subtle neuropathology (12,19).

Life span and clinical course

A group of double mutant, \(\text{NPC1}^{-/-}\text{GalNAcT}^{-/-}\), mice were followed and compared with a group of age-matched NP-C (\(\text{NPC1}^{-/-}\text{GalNAcT}^{+/+}\)) model mice (Fig 2). A conspicuous phenotypic feature of the NP-C mouse is an abnormally small body size, a feature shared by the double mutant mice. The \(\text{GalNAcT}^{-/-}\) single mutant mice were of normal size. Both \(\text{NPC1}^{-/-}\text{GalNAcT}^{-/-}\) and \(\text{NPC1}^{-/-}\text{GalNAcT}^{+/+}\) mice displayed a similar disease course. At \(-5\) weeks after birth, both types of mice began to display dyscoordination and jerky movements when walking. Prior to their deaths, both types of mice exhibited hind limb paresis, a notable neurological manifestation of the NP-C model. The mean life span of a group of nine single mutant NP-C mice was 79 days spanning days 58–96 after birth. For a similarly sized group of double mutant mice, the mean life span was 69 days, with demise between 39 and 83 days after birth. The life span of double mutant mice was not significantly different from that of the NP-C mice (\(p = 0.156\)).

Pathology

The pathology of visceral organs was similar in the NP-C mice and the double mutant mice. In both, many foamy storage macrophages, which stained lightly with periodic acid Schiff (PAS), were identified in liver, lung and spleen. The foamy macrophages within hepatic sinusoids demonstrated positive filipin staining (Fig. 3D), which has been shown to indicate the presence of cholesterol storage in NP-C cells (1). In the kidney of both types of mice, the proximal renal tubular epithelium contained clear vacuoles surrounded by PAS-positive material (not shown).

In the CNS, however, a consistent difference in neuronal storage pathology was observed between the NP-C and double mutant mice. Throughout the cerebrum, cerebellum, brainstem and spinal cord of NP-C mice, PAS-positive storage material was found within neurons. Storage was particularly pronounced in pyramidal neurons of the cerebral cortex (Fig. 4A) and in neurons of the CA3 and 4 regions in the hippocampus (not shown). Purkinje cells and spinal anterior horn cells also contained moderate amounts of storage material (not shown). In addition, many small cells, provisionally identified as microglia/macrophages, were observed throughout the CNS.

In each of the double mutant mice evaluated, neuronal storage pathology was much less conspicuous than in NP-C mice. Neurons containing PAS-positive storage material were reduced in the double mutant mice (Fig. 4B). In \(1\ \mu\text{m}\) sections of cortex, storage inclusions were obvious in the NP-C neurons, while in the double mutant mice the majority of cortical neurons were without inclusions (Fig. 4D, E and F). Staining with antibody to GM2 ganglioside revealed neuronal ganglioside storage in NP-C mice that was absent in the double mutant mice (not shown). At the ultrastructural level, neurons from the NP-C mice contained abundant small lamellar inclusions (Fig. 5A). In neurons from the double mutant mice these lamellar inclusions were rarely found (Fig. 5B). Neurons of NP-C mice exhibited punctate staining with filipin suggesting some degree of cholesterol accumulation. Interestingly, neurons of the double mutant mice did not stain with filipin (Fig. 4A).

Although the degree of neuronal storage appeared to be reduced in the double mutant mice, other aspects of their CNS pathology remained similar to the NP-C model. These included the presence of storage microglia/macrophages, the characteristic Purkinje cell loss and demyelination.

Lipid analysis

Brain gangliosides of NP-C mice included abnormally high levels of GM2 and GM3 in addition to the normally occurring

![Figure 1. Partial synthesis pathway of GSLs and its disruption in \(\text{GalNAcT}^{+/+}\) mice. Note, the gene encoding \(\text{GalNAcT}\) is known as \(\text{Galgt1}\) and was formally known as \(\text{Ggm2}\).](Image 48x617 to 281x747)

![Figure 2. Life span of NP-C and \(\text{NPC1}^{-/-}\text{GalNAcT}^{-/-}\) double mutant mice. Each group contained nine mice.](Image 347x588 to 514x747)
complement of complex ganglioside species (Fig. 6). As described previously, brain gangliosides of GalNAcT–/– single mutant mice consisted mainly of the simple gangliosides, GM3 and GD3. A substantial portion of the GD3 was found to be O-acetylated (Fig. 6). The brain ganglioside profile of the double mutant mice was indistinguishable from that of the single mutant GalNAcT–/– mice (Fig. 6) showing that accumulation of ganglioside GM2 was completely eliminated.

A very abnormal profile of neutral brain glycolipids profiles was observed in the NP-C mice, with a significant increase of glucosylceramide and lactosylceramide, as well as of GA2 and GA1 (Fig. 6). The only abnormality in the mutant GalNAcT–/– mice was an increase of lactosylceramide, to a level very similar to that observed in the NP-C mice. In the double mutant mice, lactosylceramide increase was the same as in the parent mice while glucosylceramide was about half the concentration.
observed in NP-C mice. As expected, the glycolipid species, GA2 and GA1, were absent. Free sphingosine levels were normal in brain of the mutant GalNAcT–/– mice, 5-fold elevated in NP-C mice and remained abnormal, with an intermediate value, in the double mutant mice (not shown). In addition, abnormally low levels of galactosylceramide (Fig. 6) were found in the brain of both NP-C and double mutant mice, together with a significant reduction of sulfatides, total cholesterol, ethanolamine plasmalogens and sphingomyelin species with very long chain fatty acids (data not shown). The content of all myelin lipids was thus decreased, consistent with the demyelinating process observed in the NP-C and double mutant mice. Normal levels of myelin lipids were found in the GalNAcT–/– single mutant mice.

DISCUSSION

The NPC1 protein is required for the proper intracellular trafficking of cholesterol obtained by endocytosis of LDL (3–6). NPC1 contains a sterol-sensing domain in accord with a role in the cellular redistribution of cholesterol. This domain is shared with other proteins involved in cholesterol homeostasis such as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and the sterol response element-binding protein cleavage-activating protein (SCAP). The subcellular location of NPC1 (20,21) in late endosome/lysosomal compartments is also consistent with its proposed function in sterol shuttling from lysosomes. Recent results have suggested that NPC1 may also be required for the intracellular movement of endocytosed material other than chole-
terol. In NP-C human fibroblasts, GM2 ganglioside accumulated in lysosomes in the absence of cholesterol (22,23), suggesting that NPC1 may direct the transport and ultimate degradation of gangliosides. Other results suggest that NPC1 may have a role in regulating the intracellular movement of many types of endocytosed cargo that pass through lysosomes (21).

Gangliosides and other glycolipids are the major stored lipids in the CNS of NP-C patients (7). One component of the storage material is GM2 ganglioside, which has been suspected of causing neuronal degeneration and dysfunction in NP-C and other storage disorders (8–11). Possible pathogenic mechanisms mediated by GM2 accumulation include physical constraints on neuronal function due to massive intracellular storage, the formation of meganeurites and the elaboration of toxic derivatives such as lyso-GM2. To determine the importance of ganglioside storage in the pathophysiology of NP-C, complex ganglioside synthesis was disrupted genetically. An identical design was used previously to ascertain the importance of ganglioside storage in the neuropathology and disease phenotype in the Sandhoff disease mouse, a GM2 gangliosidosis model (12). Like the Sandhoff disease paradigm, the NP-C mice showed an impressive alleviation of their neuronal storage pathology in the absence of ganglioside synthesis. However, unlike the Sandhoff model, where the life span was increased considerably in the absence of GM2 storage, the double mutant NP-C mice showed no life span increase or noticeable clinical improvement.

Neuronal storage is one of several characteristic features of CNS pathology in NP-C. Although neuronal storage was reduced in the double mutant NP-C mice, other aspects of NP-C pathology remained. Similar levels of foamy macrophages/microglia were found in the brain in the NP-C and double mutant mice. Demyelination continued to be a prominent feature in the double mutant mice. Neurodegeneration as determined by the frequency of apoptotic cells and the loss of Purkinje cells was also not curtailed by the elimination of complex ganglioside synthesis.

Our findings raise the possibility that the pathology and neurological disease in the NP-C mice may be influenced by the accumulation of lipids not affected by the GalNAcT mutation. Storage lipids that remained in the double mutant mice included GM3, lactosylceramide, glucosylceramide and free sphingosine. Although no overall storage of cholesterol occurs in NP-C brain (24), some neuronal accumulation was detected by filipin staining. This storage appeared to be reduced in the double mutant mice, suggesting that the neuronal accumulation of this lipid is not important in neuropathogenesis.

Substrate deprivation therapy is an approach for treatment of the glycolipid storage diseases using glycosphingolipid inhibitors to prevent their synthesis and subsequent accumulation (25). NB-DNJ, an inhibitor of glucosylceramide synthesis, the precursor to most gangliosides, has been shown to be effective in reducing the load of CNS ganglioside storage in mouse models of the GM2 gangliosidoses (26,27). If accumulation of small glycosphingolipids is an important factor for CNS degeneration in NP-C disease then substrate deprivation therapy using NB-DNJ may be a potential therapeutic approach.

It is not known to what extent visceral storage and pathology might affect CNS manifestations in NP-C. For instance, it is possible that toxic, water-soluble metabolites produced in the visceral organs may enter the circulation, cross the blood–brain barrier and contribute to neuronal dysfunction in NP-C. Since there was no noticeable pathological improvement in visceral organs of the double mutant mice, an indirect impact on CNS function via visceral storage cannot be ruled out.

Currently there is no therapy that can retard or curtail the NP-C disease process. It is essential to understand the pathophysiological process in NP-C in order to identify potential therapeutic targets. A genetic approach, as we have described, to delete specific metabolites or other potential pathogenic factors can be of great utility in unraveling the neurodegenerative pathway in NP-C and other lipidoses.

**MATERIALS AND METHODS**

**Establishment of NPC−/− GalNAcT+/− mice**

Heterozygous BALB/cNctr-npc1+ mice were bred with GalNAcT+/− mice to obtain NPC1+−GalNAcT+/− mice. The doubly heterozygous mice bred and NPC1+/−GalNAcT−/− mice were obtained at the expected Mendelian frequency. To minimize variations due to genetic background differences all genotypes used in this study were derived from these cross-breeding. The NPC1 mutation was identified by PCR. The GalNAcT-targeted disruption was identified by Southern blotting. Mice were observed to note general clinical condition. When mice were no longer unable to take food or water they were humanely sacrificed.

**Histology**

Four NPC1−/−GalNAcT−/− (three at 2 months, one at 3.25 months), two GalNAcT+/−NPC−/− (2 months old), two GalNAcT−/−NPC1+/− (one at 4 months, one at 2 months) and four wild-type mice (three at 2 months, one at 3.25 months) were used. The tissues were processed for paraffin or frozen sections. Paraffin sections were cut 5 µm thick. Central and peripheral nervous tissues were stained with hematoxylin & eosin (H&E), solochrome & eosin, and Luxol Fast Blue (LFB)–PAS. Visceral organs were stained with H&E and PAS stains. Coronal sections of the cerebrum, sagittal sections of the cerebellum and transverse sections of the brainstem and spinal cord were stained with either filipin (40 µm thick) or PAS (8 µm thick). Frozen sections were stained with antibody to GM2 ganglioside as described (12). For electron microscopic analysis tissues were processed for embedding in plastic resin. Sections (1 µm thick) were cut and stained with toluidine blue. Appropriate areas for further thin sectioning were selected under light microscopy and trimmed for ultrathin sectioning. Ultrathin sections were double stained with uranyl-acetate and lead citrate and examined with a Zeiss 910 electron microscope.

**Lipid analysis**

Brain and liver from 64-day old mice of all the four genotypes were available for lipid studies. Samples had been kept frozen at −25°C or lower prior to analysis, in tight containers to avoid dehydration. Total lipids were obtained as described previously (28). An aliquot of the total lipid extract was desalted and fractionated into a neutral lipid fraction and an acidic lipid fraction using Bond Elute C18 columns (Varian, Palo Alto, CA) (29). Gangliosides were analyzed without further purification using the acidic lipid fraction (28). For brain tissue, the
neutral lipid fraction corresponding to ~150 mg wet tissue was subjected to the mercuric chloride–saponification procedure to remove glycerophospholipids, and further fractionated on a 1 g silicic acid column (Bio-Rad, Hercules, CA, 200–400 mesh). In the chloroform–methanol 9:1 eluate, glucosylceramide was separated from galactosylceramide on borate-impregnated TLC plates. For liver tissue, the neutral lipid fraction was saponified without a prior mercuric chloride step and tested without further fractionation. Thin layer chromatography was performed using high performance thin-layer plates developed in chloroform–methanol–water (65:25:4 v/v/v) for separation of neutral and gangliosylceramides and chloroform–methanol–0.2% CaCl2 (55:45:10, v/v/v) for gangliosides. Lipids were visualized by anisaldehyde (general), orcinol-sulfuric acid (neutral glycolipids) and chloroform–methanol–ammonia (65:25:4 v/v/v) for separation of glucosyl- and galactosylceramide, and chloroform–methanol–0.2% CaCl2 (55:45:10, v/v/v) for gangliosides. Lipids were visualized by anisaldehyde (general), orcinol-sulfuric acid (neutral glycolipids) or resorcinol-chlorhydric acid (gangliosides) sprays and heating. Free sphingosine was determined by a HPLC procedure (30).

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