Critical role for glycosphingolipids in Niemann-Pick disease type C

Mark Zervas*‡, Kyra L. Somers†, Mary Anna Thrall†, and Steven U. Walkley*

Niemann-Pick type C (NPC) disease is a cholesterol lipidosis caused by mutations in NPC1 and NPC2 gene loci [1]. Most human cases are caused by defects in NPC1 [2], as are the spontaneously occurring NPC diseases in mice [3] and cats [4]. NPC1 protein possesses a sterol-sensing domain [1–3] and has been localized to vesicles that are believed to facilitate the recycling of unesterified cholesterol from late endosomes/lysosomes to the ER and Golgi [1, 5–7]. In addition to accumulating cholesterol, NPC1-deficient cells also accumulate gangliosides and other glycosphingolipids (GSLs), and neuropathological abnormalities in NPC disease closely resemble those seen in primary gangliosidoses [1, 8–12]. These findings led us to hypothesize that NPC1 may also function in GSL homeostasis [9]. To evaluate this possibility, we treated murine and feline NPC models with *N*-butyldeoxynojirimycin (*N*B-DNJ), an inhibitor of glucosylceramide synthase, a pivotal enzyme in the early GSL synthetic pathway [13, 14]. Treated animals showed delayed onset of neurological dysfunction, increased average life span (in mice), and reduced ganglioside accumulation and accompanying neuropathological changes. These results are consistent with our hypothesis and with GSLs being centrally involved in the pathogenesis of NPC disease, and they suggest that drugs inhibiting GSL synthesis could have a similar ameliorating effect on the human disorder.

Addresses: *Department of Neuroscience, Rose F. Kennedy Center, Albert Einstein College of Medicine, 1410 Pelham Parkway South, Bronx, New York 10461, USA. †Department of Pathology, Colorado State University, Fort Collins, Colorado 80523, USA.

Present address: ‡Howard Hughes Medical Institute and Developmental Genetics Program, Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, New York 10016, USA.

Correspondence: Steven U. Walkley
E-mail: walkley@aecom.yu.edu

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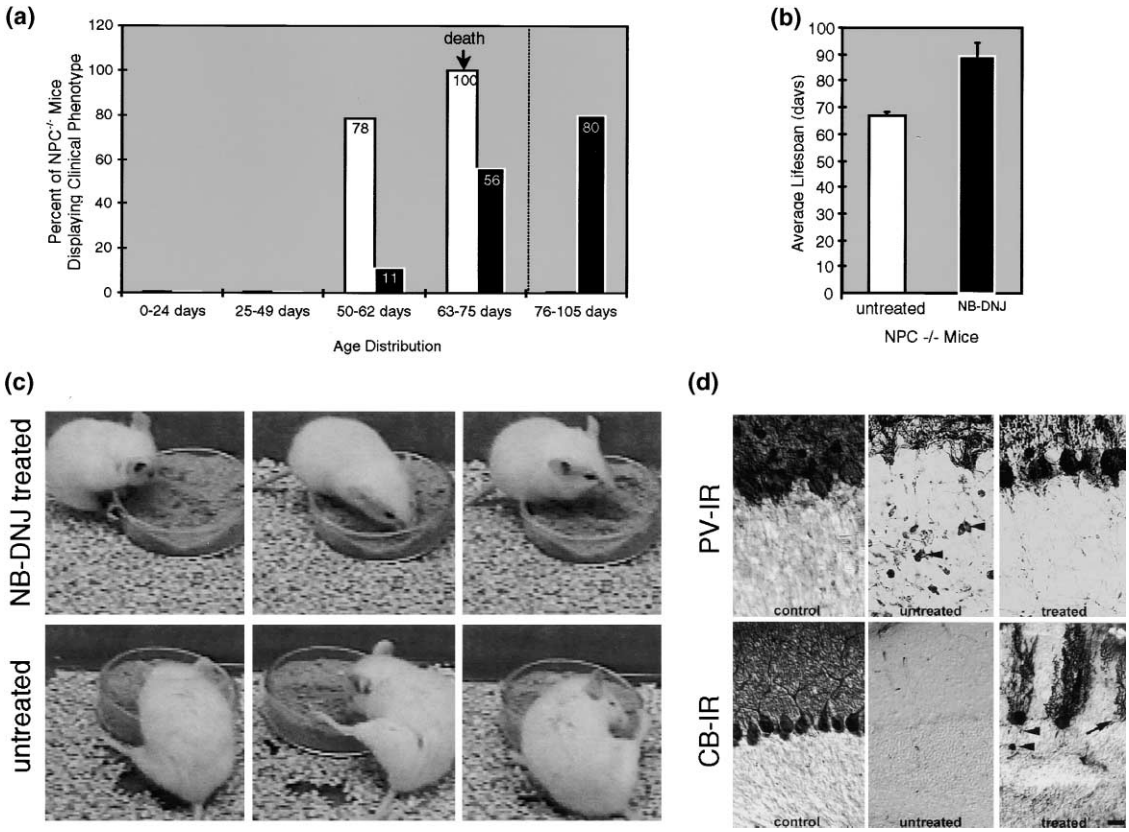
Results and discussion

Niemann-Pick type C disease is widely viewed as a disorder of cholesterol transport and homeostasis [2, 3, 15–18], but substantial evidence also exists for significant involvement of GM2 and GM3 gangliosides and other GSLs [8–11]. In order to evaluate the contribution of GSLs to NPC pathogenesis, we treated NPC-affected mice and cats with an inhibitor of glucosylceramide synthase, the enzyme responsible for the conversion of glucosylceramide to lactosylceramide, a critical early step in the synthesis of gangliosides. We provided *N*B-DNJ [13, 14] mixed in ground chow to mice (1200 mg/kg/day, beginning at 3.5 weeks of age) and by direct oral administration twice daily to cats (as given below). An assessment of the clinical phenotype of mice with NPC (n = 11) versus wild-type littermates (n = 9) revealed that in the early stages of the disease, up to 49 days of age, NPC mice did not display evidence of neurological dysfunction and were indistinguishable from wild-type littermates (Figure 1a). However, between 50 and 62 days of age, 78% of untreated NPC mice (n = 9) could be classified as having a clinical phenotype consisting of abnormal head and body tremors on movement (tremors of intention) and a pronounced ataxic gait. In contrast, we found that only 11% of NPC mice receiving *N*B-DNJ (n = 9) displayed this clinical phenotype at this age (Figure 1a). Between 63 and 75 days of age, all of the untreated mice (100%) displayed tremors and ataxia, with none living past this time period, whereas only 56% of the treated NPC mice displayed abnormal motor function during this same period (Figure 1a,c). We continued to follow a group of treated mice (n = 5) from 76 to 105 days of age, during which time 80% eventually developed the clinical phenotype (Figure 1a).

We also administered *N*B-DNJ to cats with (n = 3) and without (n = 4) NPC disease. Compiled data from untreated NPC cats (n = 10) from this colony revealed that intention tremor appears at 9.5 ± 0.3 weeks (average ± SEM), mild ataxia at 14.5 ± 0.3 weeks, and ataxia with falling at 21.5 ± 1.6 weeks. We initiated treatment of the first NPC cat at 21 weeks of age and administered *N*B-DNJ for 54 days over an 84 day period, during which time the dose was tapered from 150 mg/kg/day to 50 mg/kg/day. This animal showed a delay in the progression of intention tremor (which was present at the time that treatment was initiated) and in the onset of ataxia with falling. We subsequently treated two younger NPC cats with 50 mg/kg/day of drug beginning at 7 and 13 weeks of age and continuing without interruption for 23 and 56 days, respectively. The younger animal failed to develop any

Figure 1



*N*B-DNJ treatment in NPC mice and cats was found to delay the onset of clinical neurological disease, to increase longevity (murine NPC), and to reduce cellular pathology in the cerebellum. **(a)** The majority of untreated NPC mice showed a well-defined clinical phenotype (ataxia and intention tremor) between 50 and 62 days of age; by 63–75 days of age, clinical neurological disease and lethality were observed in all untreated NPC mice (open bars). In contrast, far fewer *N*B-DNJ-treated mice showed the clinical phenotype, and all were viable within this time frame (solid bars). NPC mice treated with *N*B-DNJ lived to 76–105 days of age, with many of them developing the clinical phenotype during this period. **(b)** An evaluation of longevity in *N*B-DNJ-treated versus untreated NPC mice showed that treated animals lived to 89 ± 5 days (average ± SEM) (solid bar) versus untreated mice, which lived to 67 ± 1 day (open bar). **(c)** Time lapse photographs of an 11-week, 5-day-old NPC mouse treated with *N*B-DNJ (upper panels) reveals a normally functioning motor system since the animal maintains itself in a stable position on its feeding dish. In contrast, a 2-week-younger, untreated NPC mouse (9 weeks, 5 days old) is unable to maintain a stable feeding position, falls to the right, and then lurches to the left (lower panels). **(d)** *N*B-DNJ treatment also reduced the characteristic cerebellar pathology that occurs in NPC disease. The cerebellar cortex from normal mice shows typical distribution of Purkinje cells and layering when it is stained with antibodies to Parvalbumin (upper left panel) and Calbindin (lower left panel). Untreated NPC mice (upper middle panel) display numerous, Parvalbumin-positive axonal spheroids of varying sizes in the granule layer (arrowheads) and regions in which all Calbindin-positive Purkinje cells have disappeared (lower middle panel). Parvalbumin staining in treated NPC mice revealed Purkinje cell axons in the granule cell layer that were mostly devoid of spheroids (upper right panel) and the presence of numerous surviving Calbindin-labeled Purkinje cells (lower right panel). The scale bar (lower right panel) represents 15 μm (applies to all).

motor system dysfunction during its treatment period, and the older animal failed to develop ataxia. The four normal cats treated with *N*B-DNJ (for 19, 23, 54, and 144 days, respectively) generally weighed less at the end of the treatment period than age-matched, untreated, normal cats but otherwise appeared healthy. *N*B-DNJ not only delayed the onset and/or progression of clinical deterioration in mice and cats but also extended the life span of mice (n = 10) from 67 ± 1 day (untreated) to 89 ± 5 days (treated), representing a 25% increase in longevity (Figure 1b).

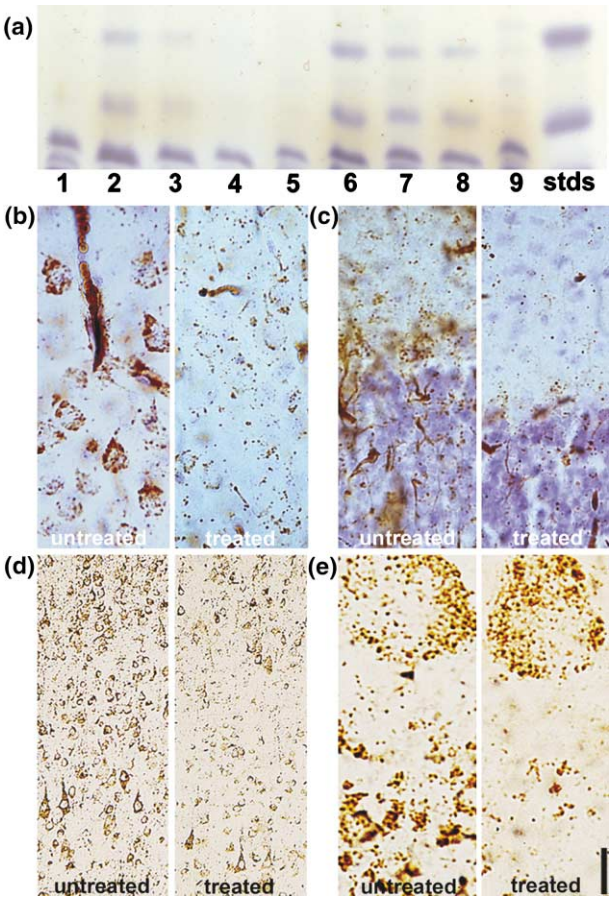
Neuropathological changes in NPC disease closely resemble those observed in primary ganglioside storage diseases [9, 12, 19]; such changes include the presence of ectopic dendrites on cortical pyramidal neurons and axonal spheroids on cerebellar Purkinje cells and other GABAergic neurons. The cerebellum is severely impacted in both the mouse and cat models of NPC and likely gives rise to a significant portion of the clinical phenotype in these models as well as in human disease [9, 12]. To evaluate the effectiveness of *N*B-DNJ therapy on cerebellar pathology, we used antibodies to Parvalbumin and Calbindin, respec-

tively, to evaluate axonal spheroid formation and Purkinje cell loss. In wild-type mice and cats, Parvalbumin-immunoreactivity was detected in Purkinje cells and in the molecular layer, while Calbindin staining was observed in Purkinje cells and their dendrites (Figure 1d). In feline NPC disease, we previously found that axonal spheroids initially appeared within the neuropil of deep cerebellar nuclei and adjacent white matter and later spread proximally toward Purkinje cell somata prior to the death of these neurons [12]. Purkinje cell axonal spheroids and death correlate with worsening motor system dysfunction in both the cat and mouse models [12, 19]. Consistent with these earlier studies, untreated 9- to 10-week-old NPC mice in the present study displayed an extensive number of Parvalbumin-positive axonal spheroids (Figure 1d). In contrast, *N*B-DNJ-treated NPC mice at this same age showed diminished spheroid formation in the granule cell layer and preserved Purkinje cell architecture to varying degrees throughout the extent of cerebellar folia. Calbindin staining of untreated NPC mice revealed a nearly complete absence of Purkinje cells in many areas of the cerebellum, while NPC mice treated with *N*B-DNJ exhibited persistence of many of these neurons (Figure 1d).

To assess the effectiveness of *N*B-DNJ in reducing ganglioside storage in NPC brain, we performed high-performance, thin-layer chromatography (HPTLC) [20] and ganglioside immunocytochemistry [9]. HPTLC analysis of NPC-affected brains confirmed GM3 and GM2 ganglioside accumulation in the cerebral cortex of mice and cats with NPC (Figure 2a). *N*B-DNJ treatment resulted in a diminution in ganglioside levels, with notable decreases of GM3 and GM2 gangliosides in the cerebral cortex of both models of NPC (Figure 2a). Semiquantitative analysis of the plates revealed that in feline and murine NPC, respectively, GM3 was reduced from 5.3% to 3.0% and from 6.3% to 4.0% of total lipid bound sialic acid, while GM2 levels were reduced from 2.7% to 1.1% and from 4.2% to 2.2% of total lipid bound sialic acid. Previous studies in which we used immunocytochemical staining to localize GM2 ganglioside in NPC disease revealed that this ganglioside was elevated in neurons of the cerebral cortex, cerebellum, and other brain regions [9]. For the present study, we made a similar analysis in treated versus untreated NPC mice (Figure 2b,c) and cats (Figure 2d,e) to determine if *N*B-DNJ treatment reduced ganglioside storage in these populations of neurons. We found that treatment caused a visible reduction in storage material within individual neurons of the cerebral cortex (Figure 2b,d) and cerebellum (Figure 2c,e) in most sections analyzed. In the cerebral cortex, GM2 immunoreactivity was most conspicuously diminished in pyramidal neurons, while in the cerebellum, GM2 was predominantly reduced in granule cells (mouse, cat) and in Purkinje cells (mouse).

Our demonstration that *N*B-DNJ ameliorates clinical neurological disease in murine and feline NPC underscores the importance of glycosphingolipid metabolism and storage in the NPC disease cascade and suggests that the inhibition of glycosphingolipid synthesis represents an

Figure 2



*N*B-DNJ treatment diminishes ganglioside storage in the brains of murine and feline NPC-affected animals. **(a)** HPTLC analysis of brain gangliosides in cats (lanes 1–4) and mice (lanes 5–9) with and without *N*B-DNJ treatment. The standards (stds) lane shows the location of GM3 and GM2 gangliosides (upper and lower spots, respectively). Untreated and treated 33-week-old wild-type cats (lanes 1 and 4, respectively), and 9.5-week-old mice (lanes 5 and 9, respectively) exhibit little or no GM3 and GM2 gangliosides, whereas age-matched, untreated cats and mice with NPC (lanes 2 and 6, respectively) show increased levels of both of these gangliosides. In comparison, the 33-week-old NPC cat treated with *N*B-DNJ for 54 days (lane 3) and the two 9.5-week-old NPC mice treated for 6 weeks (lanes 7 and 8) exhibited reduced levels of both GM3 and GM2 gangliosides. **(b–e)** Representative examples of (b,d) cerebral cortex and (c,e) cerebellum immunostained for GM2 ganglioside in NPC (b,c) mice and (d,e) cats with and without *N*B-DNJ treatment. Tissues are from the same animals used in the HPTLC studies. Untreated NPC mice and cats display GM2 accumulation in pyramidal neurons of the cerebral cortex and in Purkinje and granule cells of the cerebellum. Tissues from animals treated with *N*B-DNJ show reduced levels of GM2 immunoreactivity. The scale bar in the lower right panel represents 100 μm for (d), 10 μm for (e), and 42 μm for (b) and (c).

effective approach toward therapy for this disorder. Interestingly, similar approaches directed at lowering cholesterol in NPC disease were reported to have no significant impact on clinical neurological progression [21, 22]. *N*B-DNJ therapy has also recently been shown to effectively reduce levels of GM1 ganglioside in circulating leukocytes in human Gaucher disease [23] as well as GM2 ganglioside levels and cytopathology in murine Tay-Sachs and Sandhoff diseases [24, 25]. Substrate deprivation has also been achieved genetically by a cross of Sandhoff disease mice (in which GM2 ganglioside accumulation occurs secondarily to a deficiency in lysosomal β-hexosaminidase) with another line of mice in which the gene for β1,4-*N*-acetylgalactosaminyltransferase (GalNAcT, or GM2 synthase) had been ablated [26]. The latter mice lack the ability to produce GM2 and higher-order complex gangliosides. The double mutant animals, in comparison to the mice with Sandhoff disease alone, exhibited significantly less intracellular storage, improved neurological function, and increased longevity. The above findings indicate that substrate deprivation is an effective therapeutic approach to reducing ganglioside accumulation in a number of storage disorders. Recently, NPC mice have also been subject to substrate deprivation through a similar genetic cross with GalNAcT knockout mice [27]. The double homozygous mutants, as anticipated, did not display any detectable accumulation of GM2 or other complex gangliosides but rather possessed higher-than-normal levels of GM3 and GD3 gangliosides. Interestingly, neurons in these animals were reported to lack evidence for an accumulation of unesterified cholesterol, a finding consistent with the possibility that cholesterol storage in NPC neurons occurs secondarily to the accumulation of GM2 or higher-order glycosphingolipids. In contrast to the Sandhoff-GalNAcT double knockouts, clinical disease in the NPC-GalNAcT double mutants did not improve; these results suggest that the storage of higher-order gangliosides and cholesterol are not essential for the generation of brain dysfunction. Notably, it has been previously shown by HPTLC analysis that neutral glycolipids in the early glycosphingolipid pathway (glucosylceramide and lactosylceramide) are also elevated in NPC disease [8, 10, 11], and of these, lactosylceramide remained equivalent to NPC-affected brain in the double mutant mice. It is therefore likely that the treatment of NPC disease with *N*B-DNJ in our study is beneficial because it diminishes glycosphingolipid synthesis at an early point in the biosynthetic pathway and thus has the potential to reduce multiple storage elements of the disease. It will be important in future studies to determine the precise influence of *N*B-DNJ on these neutral glycolipids as well as on cholesterol storage.

In summary, we have shown that the glucosyltransferase inhibitor, *N*B-DNJ, is effective in reducing ganglioside accumulation, cellular pathology, and clinical neurological progression in murine and feline NPC disease. While it is conceivable that the beneficial effect of *N*B-DNJ is unrelated to its effect on GLS synthesis, a similar result stemming from the use of a related but more specific glucosylceramide synthase inhibitor, *N*-butyl-deoxygalactonojirimycin, or *N*B-DGJ (14), on NPC mice (Gondré-Lewis and S.U.W., unpublished data) supports the role for GSLs. Our results are consistent with the view that NPC1 is in some manner linked to ganglioside trafficking or synthesis and that GSLs are centrally involved in the NPC pathogenic cascade. Successful amelioration of NPC disease in murine and feline models also suggests that inhibitors of GSL synthesis may be useful in the clinical management of human NPC disease.

Materials and methods

Reagents and models

*N*B-DNJ (OGT 918; Vevesca) was generously provided by Oxford GlycoSciences. Anti-GM2 ganglioside antibody (1:2,000) was kindly provided by P. Livingston (Memorial Sloan Kettering Medical Center, New York). Anti-Parvalbumin antibody (1:5,000 and 1:10,000 on cortical and cerebellar sections, respectively) and anti-Calbindin antibody (1:500) were purchased from Sigma (St. Louis, Missouri, USA). The BALBc/NPC^{nih} mice [11] used to establish a breeding colony were obtained from Dr. Peter Pentchev at the NIH (Bethesda, Maryland, USA), while cats with NPC [28] were from a colony of animals maintained at Colorado State University. For phenotype assessment, mice were evaluated based on the presence or absence of standardized clinical features: The presence of both an ataxic gait and an intention tremor was scored as displaying a clinical phenotype. Two separate trials in mice were done. The first was not blinded; the second trial was blinded and supported the results of the first trial. An assessment of the neurological states of the cats utilized standard postural, reflex, and cranial nerve evaluations performed by a veterinary neurologist lacking knowledge of the treatment status of the animals. Procedures for which animals were used were performed in accordance with the recommendations and approval of the Institutional Animal Care Use Committees of the Albert Einstein College of Medicine and Colorado State University.

HPTLC and Immunocytochemistry

For HPTLC studies, frozen tissue was homogenized, and total lipids were obtained by standard Folch extraction. A volume of total lipid extract equivalent to 10 mg wet weight was base partitioned via published methods [20]. The upper phase was purified by SEP PAK C_{18} chromatography, dried under N_2, and resuspended, and the extract was separated on HPTLC plates. Total lipid bound sialic acids (which include gangliosides) were imaged with resorcinol and identified by comparison with purified ganglioside standards. Developed HPTLC plates were scanned with an AGFA desktop scanner; HPTLC semiquantitative analysis was done with NIH Imaging 1.62. GM3 and GM2 levels fell within a linear range when compared to a calibration curve determined by a scan of known quantities of GM1 ganglioside. Immunocytochemical methods used in these studies were carried out as previously reported [9].

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