Genetic variations and treatments that affect the lifespan of the NPC1 mouse

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Abstract **Niemann-Pick type C (NPC) disease is a multisystem disorder caused primarily by a mutation in the *npc1* gene. These studies evaluated the effect of genetic background, deletion of additional genes, and administration of several agents on the age at death in a murine model of this disorder. Such factors as differing strain background or genetic drift within a given background in the *npc1*$^{-/-}$ mouse significantly altered the age at death and the degree of organ disease. Genetic deletion of *Siat9* (GM3 synthetase) or *Nr1h2* [liver X receptor (LXR)β] shortened the life of the *npc1*$^{-/-}$ animals. Daily treatment of the *npc1*$^{-/-}$ mice with an LXR agonist or administration of a single dose of cyclodextrin, with or without the neurosteroid allopregnanolone, significantly slowed neurodegeneration and increased the lifespan of these animals. These data illustrate that the age at death of the *npc1*$^{-/-}$ mouse can be significantly influenced by many factors, including differences in strain background, other inactivating gene mutations (*Siat9* and *lxrβ*), and administration of agents such as LXR agonists and, particularly, cyclodextrin. It is currently not clear which of these effects is nonspecific or which might relate directly to the molecular defect present in the NPC1 syndrome.—** Liu, B., H. Li, J. J. Repa, S. D. Turley, and J. M. Dietschy. **Genetic variations and treatments that affect the lifespan of the NPC1 mouse. *J. Lipid Res.* 2008.** 49: **663–669.**

Supplementary key words cyclodextrin • allopregnanolone • neurodegeneration • nuclear receptors • lysosomes • gangliosides • Purkinje cells • Niemann-Pick type C1 disease

Niemann-Pick type C (NPC) disease is one of a number of lysosomal storage diseases and results primarily from a mutation that inactivates the protein NPC1 that is responsible for the movement of unesterified cholesterol from the late endosomal/lysosomal compartment to the cytosol in every cell (1). As a result, cholesterol accumulates in virtually all tissues in the body, causing organ dysfunction that may manifest clinically as hepatosplenomegaly, prolonged neonatal jaundice, liver dysfunction, pulmonary failure, and, ultimately, progressive neurological dysfunction secondary to selective neurodegeneration. These clinical findings are reproduced in a murine model of this disease that also arose as a spontaneous mutation in the *npc1* gene (2, 3). In mice homozygous for this mutation, and with a BALB/c genetic background, the concentration of cholesterol becomes increased with age in nearly every organ, and there is neonatal cholestasis, liver cell death, pulmonary dysfunction, and selective nerve cell death (4–8). Despite this block in intracellular sterol movement, however, an increased rate of cholesterol synthesis within the cytoplasmic compartment allows for essentially normal rates of plasma membrane sterol turnover, bile acid synthesis, and steroid hormone production (9, 10).

This murine model has proved very valuable as an experimental animal in which to explore the effects of various genetic and pharmacological manipulations in an attempt to better understand the pathophysiology of this disorder. The effect of such manipulations can be assessed in these animals by evaluating a number of end points, such as liver function tests, pulmonary diffusion capacity, the level of macrophage infiltration in tissues, mRNA levels for different inflammatory proteins, clinical neurological function, and quantitation of specific nerve cell numbers. Despite the availability of these many precise end points, the age at death of these animals continues to be used as a relatively easy measure of the effects of experimental manipulations that might alter the genetic defect in this disease. However, we recently found that many manipulations, some of which probably do not relate directly to the defect in cholesterol transport, can alter the age at which the *npc1*$^{-/-}$ mouse dies. If this is the case, then changes in the age at death could lead to erroneous conclusions with respect to the molecular events dictating the pathophysiology of NPC disease. This report outlines a number of factors affecting the lifespan of the *npc1*$^{-/-}$ mouse, including genetic drift in the colony, changes in the strain background of the mutant animals, deletion of the function of addi-

Manuscript received 15 November 2007 and in revised form 5 December 2007.

Published, JLR Papers in Press, December 12, 2007.
DOI 10.1194/jlr.M700525-JLR200

Abbreviations: LDLR, low density lipoprotein receptor; LXR, liver X receptor; NPC, Niemann-Pick type C; UTSW, University of Texas Southwestern.

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tional genes, and treatment with several agents that may alter the natural history of this disease. These studies suggest the potential hazards inherent in interpreting the relevance of various treatments to overcoming this genetic defect based on changes in the age at death of the experimental animals.

MATERIALS AND METHODS

Animals

These studies were undertaken using eight groups of genetically modified mice. One group of animals lacking functional NPC1 protein ($npc1^{-/-}$) on a BALB/c background was derived from heterozygous animals originally obtained from the National Institutes of Health 9 years ago (2, 3) and maintained in the animal colony at the University of Texas Southwestern (UTSW) Medical School. A second group of similar $npc1^{-/-}$ mice was derived from heterozygous founders purchased from the Jackson Laboratories (BALB/cNctr-$Npc1^{m1N}$/J; stock number 003092) this year. Animals from the original UTSW colony were also crossed with low density lipoprotein receptor-deficient ($ldlr^{-/-}$) (11), $Siat9^{-/-}$ (12), liver X receptor β-deficient ($lxrβ^{-/-}$) (13), and $abca1^{-/-}$ animals (14) to yield double knockouts designated $npc1^{-/-}/ldlr^{-/-}$, $npc1^{-/-}/Siat9^{-/-}$, $npc1^{-/-}/lxrβ^{-/-}$, and $npc1^{-/-}/abca1^{-/-}$, respectively. In each case, the appropriate control $npc1^{-/-}$ littermates having the same respective, mixed strain backgrounds were derived and are designated $npc1^{-/-}/ldlr^{+/+}$, $npc1^{-/-}/Siat9^{+/+}$, $npc1^{-/-}/lxrβ^{+/+}$, and $npc1^{-/-}/abca1^{+/+}$. In nearly all cases, these various crosses occurred over >10 generations. After these crosses, the female mice were maintained on a low-cholesterol (0.03%, w/w) rodent diet (No. 7002; Harlan Teklad, Madison, WI) during the pregnancies. The *npc1* genotype was determined by a PCR method (2), and mice exhibiting aberrant lifespans relative to similarly treated animals (outliers) had their genotypes confirmed at the time of death. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the UTSW Medical School.

Treatments

All mice were weaned at 19 days of age and fed ad libitum a low cholesterol (0.02%, w/w) rodent diet (No. 7001; Harlan Teklad) or the ground meal form of this same diet containing either cholesterol (1%, w/w) or an LXR agonist (T0901317; Cayman Chemical). Intake of this latter diet provided each animal with an approximate daily dose of T0901317 equal to 50 mg/kg body weight. Based on a previous publication (15), other groups of mice were administered a single subcutaneous injection at the scruff of the neck at 7 days of age of a 20% (in saline) solution of 2-hydroxypropyl-β-cyclodextrin (4,000 mg/kg body weight) with either 5.6 degrees of substitution (Aldrich; product 332607) or 4.5 degrees of substitution (Sigma; product H107). These values indicate the average number of hydroxypropyl groups per cyclodextrin molecule. In addition, in some experiments, allopregnanolone (5α-pregnan-3α-ol-20-one) (Sigma; product P8887) was added to the cyclodextrin solutions at a concentration of 1.5 mg/ml (to provide 25 mg/kg body weight) as described (15).

Tissue cholesterol concentration, liver function tests, and cerebellar histology

In one experiment, 56-day-old animals were used to measure the weight and cholesterol concentration in different organs, and plasma was obtained to measure liver function tests (8, 16). In another experiment, samples of the anterior cerebellum were taken for histological examination.

Animal monitoring

The general clinical condition of the mice was monitored daily. Once the mice began to show difficulty accessing the pelleted basal diet, they were also provided access to a powdered form of this diet. When mice were no longer able to take food or water, they were humanely euthanized, and this was considered the day of death.

Statistical analysis

All numeric results are expressed as means ± SEM for each treatment group. GraphPad Prism software (GraphPad, San Diego CA) was used to perform all statistical analyses. To compare multiple groups, a one-way ANOVA with Neumann-Keuls post hoc comparison was performed. For comparison of only two groups, a Student's *t*-test was used. In all cases, statistically significant differences were declared at $P < 0.05$.

RESULTS

Effects of genetic drift and strain background on age at death of $npc1^{-/-}$ mice

Although the UTSW and Jackson colonies of NPC mice were derived from the same source, they had been isolated from one another for ~9 years. Whereas both colonies were maintained on a BALB/c background, $npc1^{-/-}$ animals from the UTSW colony lived significantly longer (89 ± 1.4 days) than those from the Jackson colony (80 ± 0.8 days) when observed during the same time period (**Fig. 1A**). However, although both groups of mice had similar body weights (**Fig. 2A**), enlarged livers (Fig. 2C), and cholesterol concentrations in the liver (Fig. 2D), spleen (Fig. 2E), and lung (Fig. 2F), the plasma transaminase levels were only increased half as much (Fig. 2H, I) in the Jackson, compared with the UTSW, animals. Thus, genetic drift in these two colonies affected both the age at death and the severity of the liver disease in these $npc1^{-/-}$ mice with the BALB/c background.

Importantly, crossing these animals into different genetic backgrounds also had significant effects on lifespan. For example, introducing the C57BL/6 and 129/SvJ backgrounds from the $ldlr^{-/-}$ animals into the BALB/c $npc1^{-/-}$ mice decreased the age at death to 79 ± 1.0 days, whereas the similar, mixed strain background derived from the $abca1^{-/-}$ mice prolonged lifespan to 91 ± 1.1 days (Fig. 1B). Also of note is the range of values seen in these 142 $npc1^{-/-}$ mice, where some animals died at 63 days of age but others reached 109 days of age (Fig. 1). Even when the strain background in a group of $npc1^{-/-}$ animals was constant, the range of ages at death varied over an interval of 20 days in some groups.

Effects of changes in sphingolipid metabolism and lipoprotein cholesterol flux on age at death

In the studies shown in **Fig. 3**, every experimental manipulation was judged against an appropriate, untreated $npc1^{-/-}$ control group with an identical genetic back-

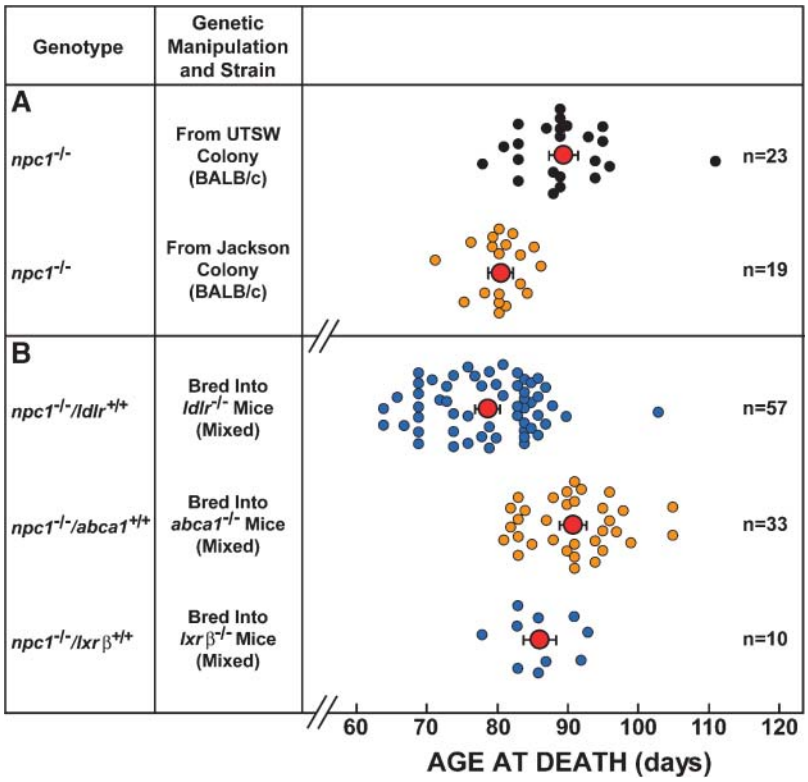

Fig. 1. Effects of genetic separation and different strain backgrounds on the age at death of Niemann-Pick type C1-deficient (*npc1*$^{-/-}$) mice. The original *npc1*$^{-/-}$ mice on a BALB/c background were obtained directly from the National Institutes of Health in 1997 to establish a colony at the University of Texas Southwestern (UTSW) Medical School. A similar colony was later established at the Jackson Laboratories. A: Age at death of 42 animals taken from these two colonies and observed simultaneously. B: The same *npc1*$^{-/-}$ mice with the BALB/c background from the UTSW colony were bred over the last 9 years with low density lipoprotein receptor-deficient (*ldlr*$^{-/-}$; C57BL/6 and 129/SvJ backgrounds), *abca1*$^{-/-}$ (C57BL/6 and 129/Ola backgrounds), and liver X receptor β-deficient (*lxrβ*$^{-/-}$; C57BL/6 and 129/SvJ backgrounds) mice to generate double knockout animals. This panel shows the age at death of the littermates of these crosses that had only the *npc1*$^{-/-}$ genotype but were of different mixed strain backgrounds. The colors have no specific meaning and are used only to separate individual mice in the various groups. The red circles show the mean ± SEM for the number (n) of animals with each genetic background.

ground. However, for the purposes of this figure, all of these 142 control *npc1*$^{-/-}$ animals were combined into a single group and normalized to a mean age at death of 85 days (Fig. 3A). Although various gangliosides also accumulate in the brains of the *npc1*$^{-/-}$ mice (17), elimination of GM3 ganglioside synthesis by deleting the *Siat9* gene significantly decreased the lifespan of the mutant mice (Fig. 3B). In contrast, there was no effect on the age at death of these *npc1*$^{-/-}$ animals when they were fed cholesterol or when LDLR activity was deleted (Fig. 3C). In the first instance, increased delivery of cholesterol carried in chylomicrons to the liver worsened the hepatic disease (8), whereas in the second instance, deletion of LDLR activity increased the delivery of cholesterol carried in LDL to the lungs and worsened pulmonary function (16). Neither manipulation, however, is known to alter cholesterol flux across the blood-brain barrier.

Effects of altered LXR function or administration of cyclodextrin on age at death

In contrast to these latter observations, deletion of LXRβ function shortened lifespan (74 ± 2.2 days), whereas stimulation of this receptor activity by administration of the agonist significantly prolonged life (93 ± 1.8 days) (Fig. 3D). Because this receptor is expressed in the brain (18), these effects could conceivably reflect changes in cholesterol flux across the central nervous system. More strikingly, a single dose of cyclodextrin given at 7 days of age markedly prolonged the average life (>108 days) of these *npc1*$^{-/-}$ mice, but the addition of allopregnanolone to this regimen had no additional effect (Fig. 3E). Although there was an ~80% reduction in Purkinje cell number in the untreated *npc1*$^{-/-}$ mice (**Fig. 4A, B**), treatment with cyclodextrin increased the number of these cells surviving at 49 days of age nearly 3-fold (Fig. 4C).

DISCUSSION

Although the age at death of the *npc1*$^{-/-}$ animal would seem to be a definitive end point to use in evaluating the effectiveness of various manipulations that might elucidate the pathogenesis of NPC disease, it is clear from these studies that many presumably nonspecific manipulations can significantly alter this end point. We found, for example, that offering the partially debilitated mouse ground food or subjecting the animal to daily exercise on a rotarod apparatus significantly prolongs the lifespan of the animal compared with the mouse offered only solid food or not subjected to exercise (data not shown). In addition to these effects of animal husbandry, the genetic background of the mice can have a major effect on the age at death even though the mutation in the NPC1 gene presumably is unchanged. For example, genetic drift in the colony (Fig. 1A) or the introduction of a new genetic background from other mouse strains (Fig. 1B) can significantly change not only the age at death but, in some cases, the severity of the clinical disease (Fig. 2) seen in the *npc1*$^{-/-}$ mouse. These observations raise several important issues with respect to performing studies in which age at death is the end point. First, the control *npc1*$^{-/-}$ mice against which the effectiveness of a particular manipula-

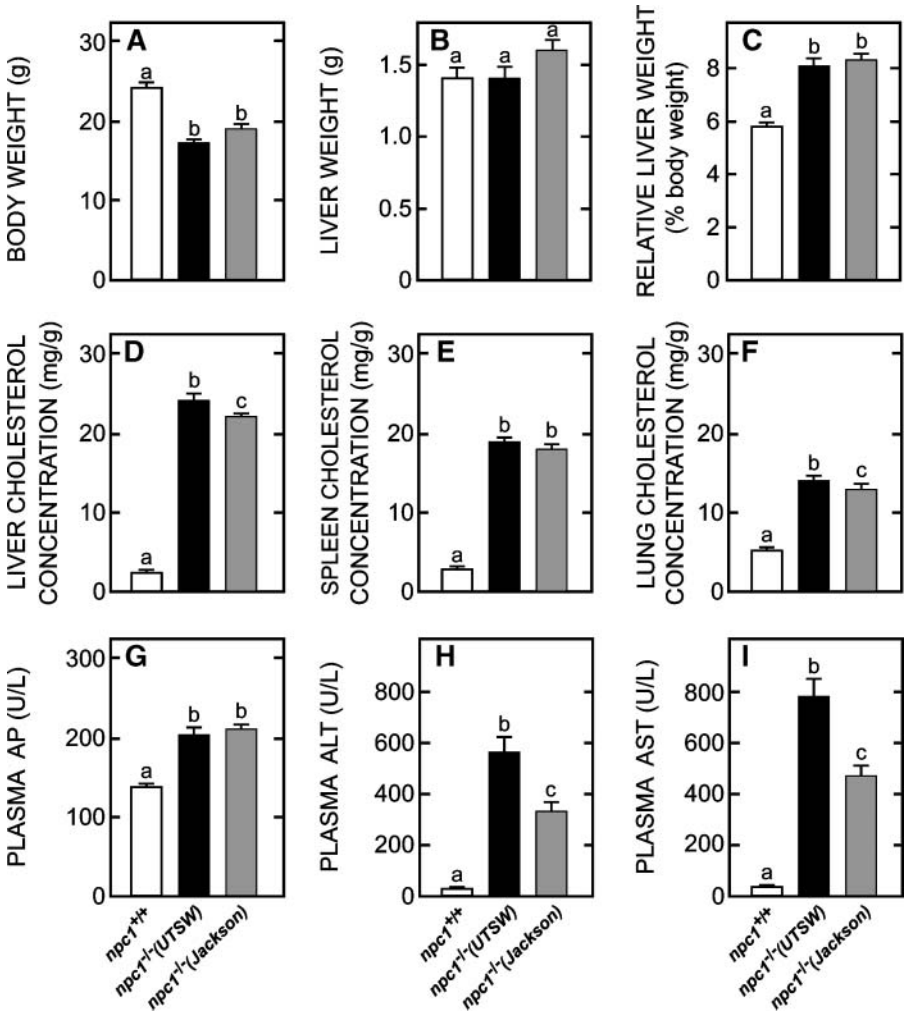

Fig. 2. Tissue cholesterol concentrations and liver function tests in mice from the UTSW and Jackson Laboratory colonies. These studies were carried out in 56-day-old $npc1^{+/+}$ and $npc1^{-/-}$ mice derived from both the UTSW and Jackson Laboratory colonies. As there were no differences in metabolic parameters in the $npc1^{+/+}$ animals that came from either colony, these two groups of mice were combined into a single group (n = 9). The groups of $npc1^{-/-}$ mice were derived from either the UTSW (n = 8) or Jackson Laboratories (n = 8) stocks and studied independently. Whole body animal weights (A) and absolute (B) and relative (C) liver weights were measured, as were the concentrations of cholesterol in the liver (D), spleen (E) and lungs (F). Alkaline phosphatase (AP; G), alanine aminotransferase (ALT; H), and aspartate aminotransferase (AST; I) levels in plasma were also measured. Values depict means ± SEM of data from eight or nine mice per group. Bars denoted by different letters are statistically different ($P < 0.05$).

tion is judged must come from the same colony of animals and must have the same genetic strain background as the treated animals. Second, it is important that issues of husbandry, diet, and exercise be identical in the control and experimental groups. Third, even when these conditions are met, the age at death commonly will vary over a range of 20 days or more (Fig. 1). Thus, to avoid misleading results, the control and experimental groups must have relatively large numbers of animals. Finally, even under the best of experimental circumstances, and when the genotype is confirmed, every large group of mice consistently has outliers (Figs. 1, 3) that deviate significantly from the remaining animals in terms of the age at death. Measurements, particularly morphological characterizations, inadvertently carried out in one or two of these outliers also could lead to serious errors of interpretation.

However, in spite of all of these potential artifacts, other observations suggest that the age at death can be altered significantly by different genetic or pharmacological manipulations that do relate to the underlying transport defect found in the cells of the $npc1^{-/-}$ mouse. For example, although the accumulation of gangliosides has been postulated to play a role in neuron death in this disorder, inactivation of the gene *Galgt1*, which is responsible for the enzyme-synthesizing GM2 gangliosides, slightly decreases the age at death of the $npc1^{-/-}$ mouse (69 vs. 79 days) (19), and inactivation of *Siat9*, which encodes the protein responsible for the synthesis of GM3 gangliosides, short-

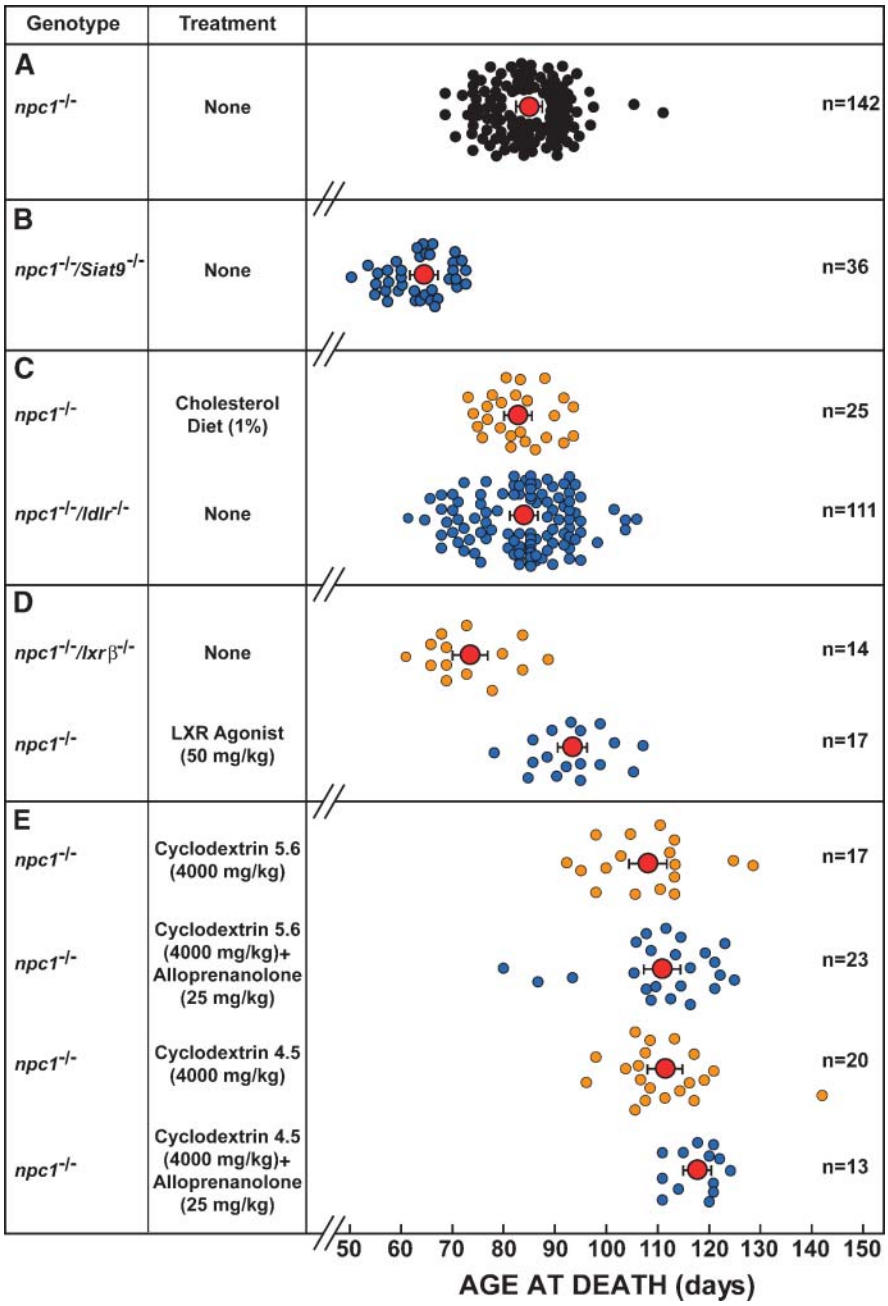

Fig. 3. Effect of various genetic deletions and dietary or pharmacological treatments on the age at death of the *npc1*$^{-/-}$ mice. This figure shows the age at death of the *npc1*$^{-/-}$ animals after nine different treatments or genetic manipulations. In each case, the treated, experimental animals were compared with untreated, control *npc1*$^{-/-}$ mice with the same strain background. For the purposes of this figure, however, the ages at death in all of these groups were normalized so that the control *npc1*$^{-/-}$ animals had a mean age at death of 85.0 days. A: Data for these combined 142 control *npc1*$^{-/-}$ mice are shown. B: Effects of deleting the activity of the enzyme GM3 synthetase (*Siat9*$^{-/-}$) that is responsible for the synthesis of GM3 gangliosides. C: Effects of either feeding a 1% cholesterol diet from weaning or deleting the LDLR are shown. D: Effects of deleting LXRβ or driving this receptor with an agonist are illustrated. E: Effects of administering a single dose of cyclodextrin 5.6 or cyclodextrin 4.5, with or without allopregnanolone, on the age at death are shown. The colors have no specific meaning and are used only to separate the individual mice in the different groups. The red circles show the mean ± SEM for the number (n) of animals in each treatment group. The manipulations in C did not significantly alter the age at death compared with the control animals in A ($P > 0.05$), whereas those treatments in B, D, and E all significantly altered the age at death ($P < 0.0001$).

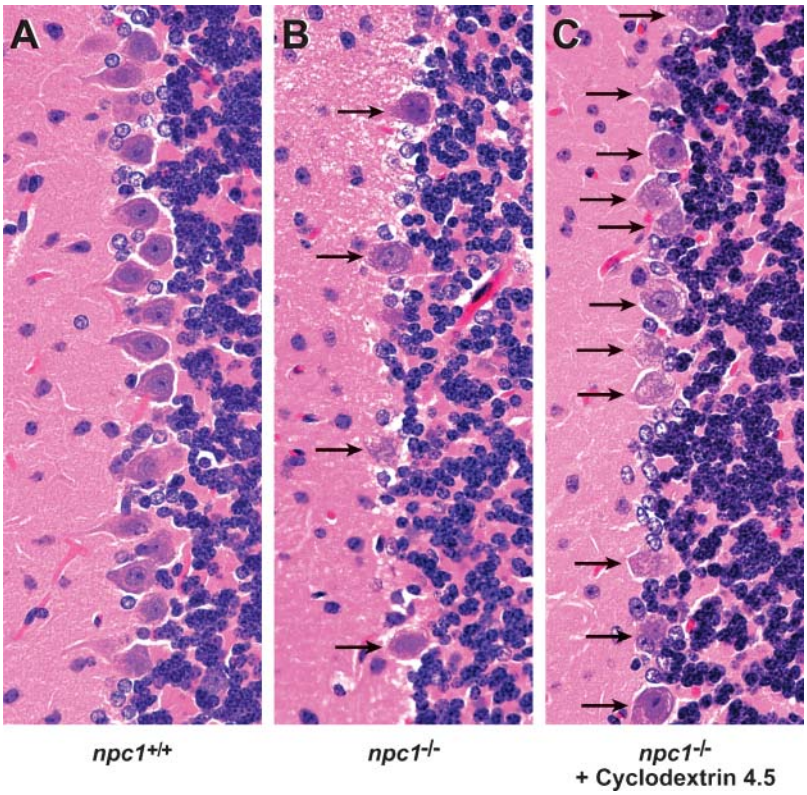

Fig. 4. Histological examination of the cerebellum of *npc1*$^{-/-}$ mice treated with cyclodextrin. Cerebella were harvested from animals at 49 days of age, and histological preparations of the anterior portion were stained with hematoxylin and eosin to show Purkinje cells. A: Tissue from *npc1*$^{+/+}$ animals injected with saline. B: Tissue from *npc1*$^{-/-}$ mice injected with saline. C: Tissue from *npc1*$^{-/-}$ animals treated with a single injection of cyclodextrin 4.5 at 7 days of age. The arrows in B and C point to surviving Purkinje cells.

ens the life expectancy of the mutant animals even more (65 vs. 85 days) (Fig. 3B). Similarly, although manipulating the amount of chylomicron cholesterol introduced into the vascular space or the level of LDL-cholesterol clearance has no effect on the age at death of these animals (Fig. 3C), manipulation of the activity of LXR, a receptor known to regulate cholesterol flux across the brain (20), clearly alters the age at death of the *npc1*$^{-/-}$ mice (Fig. 3D). Finally, another group of molecules known to alter cholesterol in the plasma membrane of cells (21), the cyclodextrins, markedly prolong the life of the mutant mice, and this effect appears to be independent of the administration of the neurosteroid, allopregnanolone. This latter observation is particularly striking because it follows the administration of the cyclodextrin as a single dose at 7 days of age. Clearly, the molecular and physiological alterations brought about by these changes in sphingolipid and cholesterol metabolism that account for the observed changes in age at death need to be investigated in detail. However, these observations emphasize that any studies that use age at death as a major end point must avoid the potential misleading results that can arise from failure to control the genetic background of the different groups of animals, failure to control different nutritional and other environmental factors affecting the animals, and, most importantly, failure to use adequate numbers of mice in the experimental groups to obtain a reliable measure of the actual time of death.

The authors thank Heather Waddell, Brian Griffith, Sean Campbell, and Chunmei Yang for their excellent technical assistance and Kerry Foreman for expert preparation of the manuscript. This work was supported by grants from the U. S. Public Health Service (Grants R01 HL-09610 and T32 DK-07745 to J.M.D. and B.L.), the Moss Heart Fund (to J.M.D.), and the Ara Parseghian Medical Research Foundation (to J.J.R.). B.L. also received postdoctoral support from a grant by the Ara Parseghian Medical Research Foundation and the Dana's Angels Research Trust.

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