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 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 colony, changes in the strain background or genetic drift, deletion of additional genes, and administration of agents such as LXR agonists and 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 Nr1h2 [liver X receptor (LXR)b]), 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

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, 5) 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-Npc1tm1N; stock number 090992) this year. Animals from the original UTSW colony were also crossed with low density lipoprotein receptor-deficient abca1 knockout mice (7), liver X receptor β-deficient (lxrb/−/−) (13), and abca1−/− animals (14) to yield double knockouts designated ldr−/−/−, npc1−/−/−/−, ldr−/−/−, and ldr−/−/−/−/−, respectively. In each case, the appropriate control npc1/−/− littermates having the same respective, mixed strain backgrounds were derived and are designated ldr−/−/−, npc1−/−/−/−/−, ldr−/−/−/−, and ldr−/−/−/−/−/−, respectively. In nearly all cases, these animals crossed 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 general clinical condition of the mice was monitored, 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 ldr−/−/− 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-

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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-

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**Table:**

| Genotype       | Genetic Manipulation and Strain          | n  |
|----------------|-----------------------------------------|----|
| *npc1* /+     | From UTSW Colony (BALB/c)                | 23 |
| *npc1* /+     | From Jackson Colony (BALB/c)             | 19 |
| *npc1* /−     | Bred Into *ldlr* /− Mice (Mixed)         | 57 |
| *npc1* /−/ldlr* /− | Bred Into *abca1* /− Mice (Mixed) | 33 |
| *npc1* /−/ldlr* /− | Bred Into *lxrβ* /− Mice (Mixed) | 10 |

**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 littersmates 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.
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 \textit{npe1}^{-/-} 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 \textit{Galgt1}, which is responsible for the enzyme-synthesizing GM2 gangliosides, slightly decreases the age at death of the \textit{npe1}^{-/-} mouse (69 vs. 79 days) (19), and inactivation of \textit{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 \( \text{npc1}^{-/-} \) mice. This figure shows the age at death of the \( \text{npc1}^{-/-} \) animals after nine different treatments or genetic manipulations. In each case, the treated, experimental animals were compared with untreated, control \( \text{npc1}^{-/-} \) 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 \( \text{npc1}^{-/-} \) animals had a mean age at death of 85.0 days. A: Data for these combined 142 control \( \text{npc1}^{-/-} \) mice are shown. B: Effects of deleting the activity of the enzyme GM3 synthetase (\( \text{Stat9}^{-/-} \)) 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 LXR\( \beta \) 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 (\( P < 0.0001 \)).
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*<sup>−/−</sup> 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, allospregnanolone. 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.

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