

# Hematopoietic cell transplantation in murine globoid cell leukodystrophy (the twitcher mouse): Effects on levels of galactosylceramidase, psychosine, and galactocerebrosides

(sphingolipid storage diseases/Krabbe disease/enzyme replacement therapy/neurochemistry/bone marrow transplantation)

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**ABSTRACT** Hematopoietic cell transplantation (HCT) prolongs survival in the twitcher mouse, an authentic animal model of human globoid cell leukodystrophy (Krabbe disease; galactosylceramidase deficiency), but the effects of HCT on levels of galactosylceramidase, psychosine, and cerebrosides in the tissues of twitcher mice have not been previously studied. Galactosylceramidase was less than 8% of control activity in tissues of untreated twitcher mice but reached normal values in brain and spleen and 20–30% of control in kidney of 100-day-old twitchers that received HCT at age 10 days. Using a recently developed method for the simultaneous determination of psychosine and cerebrosides, we measured the tissue levels of these lipids in the above animals. The levels of psychosine in brain, sciatic nerve, and kidney of untreated twitcher mice were 44, 200, and 12 times control values, respectively, in 30-day-old animals and 69, 500, and 14 times control levels in 40-day-old mice. On the other hand, levels of cerebroside were approximately 35% of control values in sciatic nerve, remained about the same in the brain, and were elevated 10-fold in the kidney of twitcher mice. After HCT, psychosine levels in the brains of 30-day-old twitchers were lowered to 30–35% of values in untreated twitchers, and the levels remained in that range during the post-HCT period. Similarly, brain cerebroside levels remained low in HCT-treated twitcher mice. Although psychosine levels in sciatic nerves of HCT-treated twitcher mice increased more slowly than in the nerves of untreated twitchers, the levels in 100-day-old HCT-treated twitcher mice had reached the same high values as those seen in untreated 40-day-old twitchers. It is not known whether the extremely high levels of psychosine in sciatic nerves ultimately contribute to the death of twitcher mice after HCT.

The precise etiology of the neuropathological damage in patients with Krabbe disease has not been elucidated, although the deficiency of galactosylceramidase (galactocerebroside galactosidase) has been established as the primary cause of the disease (1). Miyatake and Suzuki (2) have advanced the hypothesis that psychosine derived from cerebroside causes the tissue damage in patients with Krabbe disease, a hypothesis supported by the finding of elevated levels of psychosine in the brains of patients with Krabbe disease (3). Recently, the twitcher mouse has been established as an authentic model for Krabbe disease (4). Elevated psychosine levels and deficient enzymic activity have been demonstrated in the brains of twitcher mice (5). Therefore, the twitcher mouse appears to be a valuable model in which the psychosine theory can be tested.

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Unlike the pathology of Krabbe disease, central nervous system involvement is relatively mild in the twitcher mouse (4, 6, 7). Instead, severe pathological changes such as edema and demyelination occur in the peripheral nervous system (8, 9). In addition, abnormal inclusions have been found in the lymph nodes and kidney of the twitcher mouse (10). In accordance with these morphological observations, significantly lowered cerebrosides in sciatic nerve (11) and highly elevated galactocerebrosides in the kidney (11, 12) of the twitcher mouse have been reported.

Affected twitcher mice die with progressive neurodegeneration by 40–45 days of age, but hematopoietic cell transplantation (HCT) from enzymatically normal congenic mice prolongs survival by 2- to 3-fold and is associated with histological evidence of remyelination (13). However, the effects of HCT on levels of psychosine and cerebrosides in tissues of the twitcher mutant have not been previously studied. To investigate the involvement of psychosine in sciatic nerves and kidney of untreated and HCT-treated twitchers, it was essential to devise a method for the determination of extremely small amounts of psychosine in tissue samples. We have developed a method that is approximately 10 times more sensitive than previously available techniques for psychosine determination (14) and also permits the simultaneous determination of cerebroside levels (T.I., Y.K., and A.M.Y., unpublished results). Using this method, we compared the levels of psychosine and cerebrosides in brain, sciatic nerve, and kidney from untreated twitcher mice and control littermates at various ages, and we also studied tissue galactosylceramidase activity. We then examined the alterations in the levels of these compounds after HCT in twitcher mice.

## MATERIALS AND METHODS

**Animals.** Breeding pairs of C57BL/6 mice that were carriers for the twitcher mutation were obtained from The Jackson Laboratory. Presymptomatic twitcher mice and enzymatically normal or heterozygous littermates were identified by assays for galactosylceramidase in aqueous homogenates of tail tips from 7-day-old offspring, as described previously (13, 15). Affected twitchers and normal littermates were prepared for HCT by irradiation at age 9

Abbreviations and trivial names: HCT, hematopoietic cell transplantation; cerebroside, *N*-acyl-*O*- $\beta$ -galactosylsphingene; psychosine, 1-*O*- $\beta$ -galactosylsphingene. Cerebrosides containing  $\alpha$ -hydroxy fatty acid (HFA) and nonhydroxy fatty acid (NFA) are designated by prefixes hydroxy- and nonhydroxy-, respectively.

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days from a  $^{137}\text{Cs}$  source at a dose rate of 120 rad/min and a total dose of 900 rad (1 rad = 0.01 gray). Twenty-four hours after irradiation, each mouse received an intraperitoneal injection of  $10\text{--}15 \times 10^6$  bone marrow and  $35\text{--}50 \times 10^6$  spleen cells that were freshly obtained from 6- to 8-week-old female enzymatically normal congenic mice. Hematopoietic engraftment after transplantation was assessed by electrophoretic analysis of blood samples for isozymes of erythrocyte glucose-phosphate isomerase 1 (GPI-1) (16); recipient animals are homozygous for the GPI-1B isozyme, while congenic C57BL/6 donors have the GPI-1A isozyme. For 2 weeks after HCT, recipient mice and their dams were housed in sterilized microisolator cages and were given standard sterilized laboratory animal chow and acidified water to which was added neomycin (500  $\mu\text{g}/\text{ml}$ ), polymyxin B (13  $\mu\text{g}/\text{ml}$ ), and trimethoprim/sulfamethoxazole (trimethoprim at 100  $\mu\text{g}/\text{ml}$ ). Untreated twitcher mice, HCT-treated twitchers, and control untreated and HCT-treated normal littermates were killed at selected ages, and samples of brain, spleen, and kidney were obtained for determination of galactosylceramide according to the methods of Suzuki (17), using tritiated galactosylceramide as the substrate (18).

**Determination of Psychosine and Cerebrosides.** Brain, sciatic nerve, and kidney were obtained from animals immediately after sacrifice. Samples of these tissues (up to 7 mg dry weight) were homogenized in 0.5 ml of ice-cold water, and an aliquot was taken for protein determination (19). Total lipids were extracted from the rest of the homogenate, and the levels of psychosine and cerebrosides were determined as will be described elsewhere. Briefly, the total lipids were treated with [ $^3\text{H}$ ]acetic anhydride, and the acetylated mixture was added to a known amount of nonradioactive *N*-acetylpsychosine as carrier and subjected to mild alkaline methanolysis. After column chromatography, the fraction containing cerebrosides and *N*-acetylpsychosine was benzooylated, and benzooylated derivatives of these lipids were isolated by thin-layer chromatography (TLC). Amounts of benzooylated cerebrosides containing nonhydroxy and hydroxy fatty acids were determined separately by densitometric scanning of the TLC plate. The amount of psychosine was determined by measuring the radioactivity in the *N*-acetylpsychosine spot, and the recovery of this lipid was also measured by densitometric scanning.

## RESULTS

**Levels of Psychosine and Cerebrosides in Tissues of Untreated Twitcher Mice.** Using the method described above, we determined concentrations of psychosine and nonhydroxy- and hydroxycerebrosides in brain, sciatic nerve, and kidney from 30- and 40-day-old twitcher mice and littermate controls. Even with intensive supportive care, twitcher mice do not survive beyond 40–45 days of age (1). Although body and kidney weights of twitcher mice were considerably lower than control values, brain weights of twitchers were only slightly lower (Fig. 1).

In agreement with our previous study (7), the levels of both nonhydroxy- and hydroxycerebrosides in the brains of twitcher mice were only slightly lower than control values (Fig. 2). On the other hand, psychosine levels in twitcher brains were 70-fold higher than in brains of control mice, as previously shown by Igisu and Suzuki (5) (Fig. 3). In contrast to brain, sciatic nerves of twitcher mice had cerebroside levels only about one-third of the levels in control littermates (Fig. 4). In sciatic nerves of 15-day-old twitcher mice, however, the levels of psychosine were over 40-fold higher than control values, and the difference further increased to over 200- and 500-fold in 30- and 40-day-old twitchers, respectively (Fig. 5). Unlike in the nervous system and as indicated by Ida *et al.* (12), in the kidneys of twitcher mice the

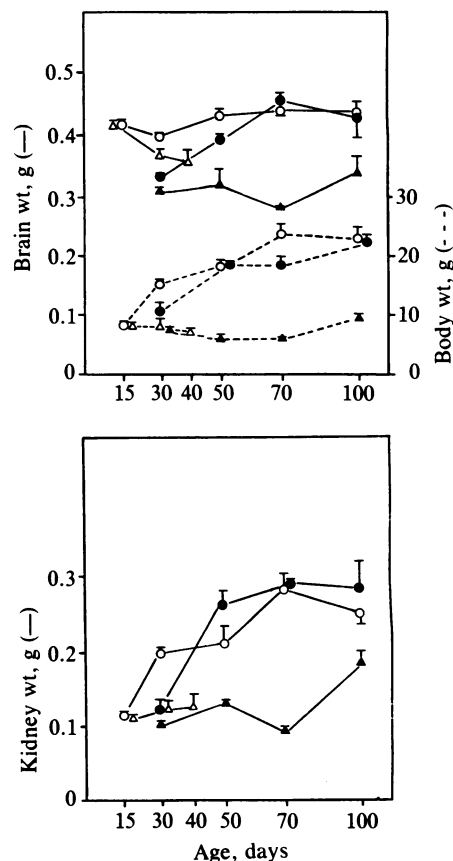


FIG. 1. Weights of body, brain, and kidney in twitcher and control mice as a function of age. Twitcher mice with or without HCT are indicated by ▲ or △, respectively. Control mice with or without transplantation are shown by ● or ○, respectively. Three to six animals were used for each experimental point. Bars indicate SEM.

levels of nonhydroxy- and hydroxycerebrosides were elevated about 10- and 100-fold, respectively, compared with control values (Fig. 6). The psychosine levels in kidneys of twitchers were also elevated by approximately 10-fold (Fig. 7).

**Effect of HCT on the Levels of Galactosylceramidase, Psychosine, and Cerebrosides.** As reported by Yeager *et al.* (13), twitcher mice survive considerably longer after receiving HCT at 10 days of age. To determine whether HCT altered levels of enzyme or glycolipid in twitchers, we

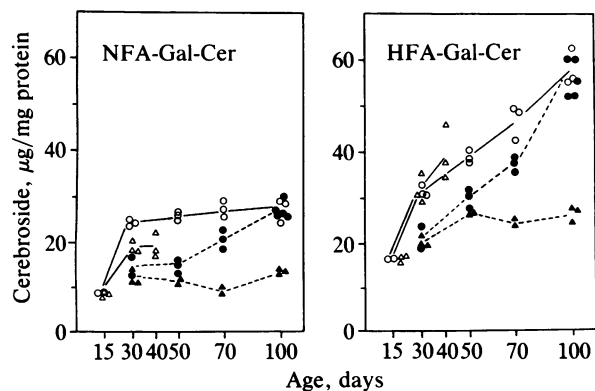


FIG. 2. Concentration of nonhydroxy- (Left) and hydroxy- (Right) cerebrosides in brains of twitcher and control mice as a function of age. See legend to Fig. 1 for the symbols. NFA, nonhydroxy fatty acid; HFA, hydroxy fatty acid; Gal, galactose; Cer, ceramide.

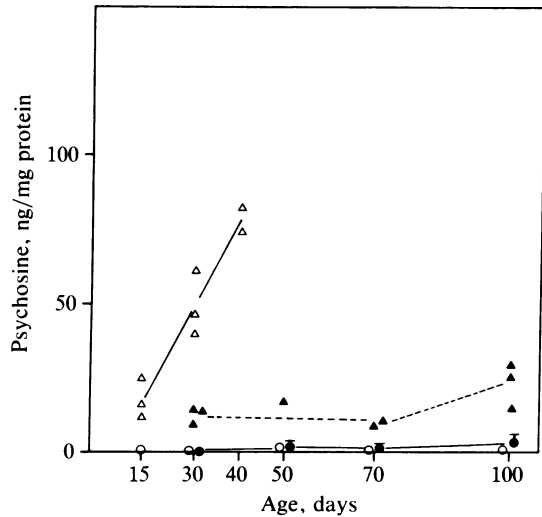


FIG. 3. Concentration of psychosine in brains of twitcher and control mice as a function of age. See legend to Fig. 1 for the symbols.

analyzed levels of galactosylceramidase in brain, spleen, and kidney from untreated and HCT-treated twitcher mice, as well as control normal littermates (with and without HCT). We also determined levels of psychosine and cerebroside in brain, sciatic nerve, and kidney from these animals.

After HCT from enzymatically normal congenic donors, galactosylceramidase in control littermates decreased in kidney of 50-day-old mice (40 days after HCT), although levels in brain and spleen remained similar to values in untransplanted normal animals (Table 1). In 70-day-old control mice (60 days after HCT), galactosylceramidase levels in all tissues had attained or exceeded enzyme activities in untreated control mice. Galactosylceramidase was barely detectable (less than 8% of control values) in tissue samples from untransplanted twitcher mice (Table 1). Gradual increases in galactosylceramidase activity were apparent in twitcher mice at 40 and 60 days after HCT (50 and 70 days of age, respectively) (Table 1), by which times enzyme levels were elevated to 15–25% of control values in brain and kidney. The most consistent and rapid increase in enzyme activity after HCT was noted in the spleen, in which galactosylceramidase levels were 30–40% of normal by 40–60 days after HCT (Table 1). By 90 days after HCT, galactosylceramidase in kidney of twitcher mice increased to only 20–30% of control values, while mean splenic enzyme activity exceeded the level observed in control normal littermates. Interestingly, normal levels of galactosylceramidase were present in twitcher mouse brains by 90 days after

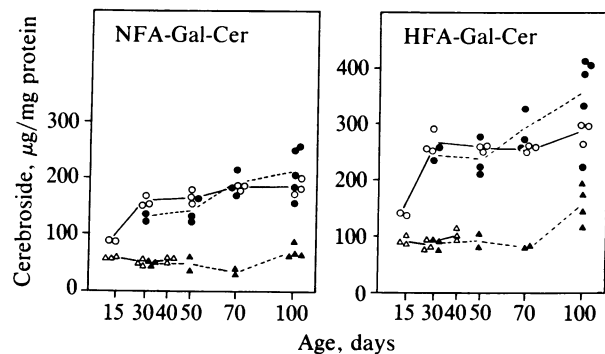


FIG. 4. Concentration of nonhydroxy- (Left) and hydroxy- (Right) cerebroside in sciatic nerves of twitcher and control mice as a function of age. See legend to Fig. 1 for the symbols.

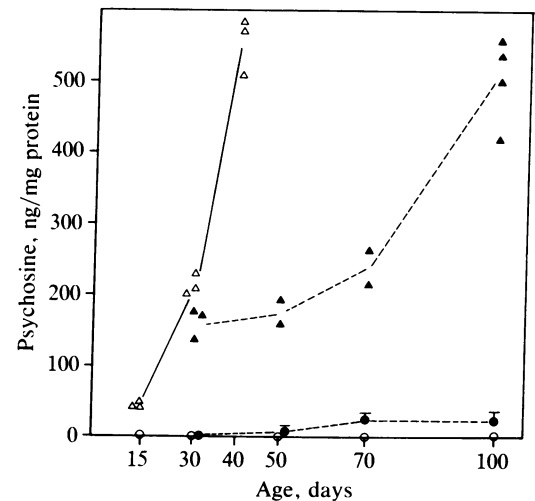


FIG. 5. Concentration of psychosine in sciatic nerves of twitcher and control mice as a function of age. See legend to Fig. 1 for the symbols.

HCT, even though no specific techniques were utilized to induce or maintain permeability of the blood-brain barrier in twitcher mice after irradiation and HCT.

Cerebroside levels in the brains of control mice that underwent HCT were significantly lower than those observed in untreated control mice at 30 days of age (20 days after HCT) (Fig. 2). However, the levels gradually increased as the animals grew older and were normal in 100-day-old mice. Cerebroside levels in the brain of transplanted twitcher mice were about the same as those of HCT-treated controls at age 30 days but did not increase with age. Psychosine levels in the brain of 30-day-old transplanted twitchers were reduced to about one-third of the values obtained in untreated twitcher mice but were still substantially higher than those of either untreated or HCT-treated controls (Fig. 3). In transplanted twitcher mice, psychosine levels in the brain remained stable until the animals reached 100 days of age, at which time the levels showed a slight additional increase.

In contrast to the observed alterations in brain, in sciatic nerve HCT appeared to have no effect on the cerebroside levels of either twitcher or control mice (Fig. 4). Similarly, psychosine levels in the sciatic nerve of 30-day-old HCT-treated twitcher mice were almost the same as in untreated twitcher mice (Fig. 5). However, the psychosine levels in sciatic nerve of transplanted twitchers increased much more slowly than in untreated animals. The levels gradually increased exponentially with age; psychosine levels in nerve obtained from 100-day-old twitcher mice that had received

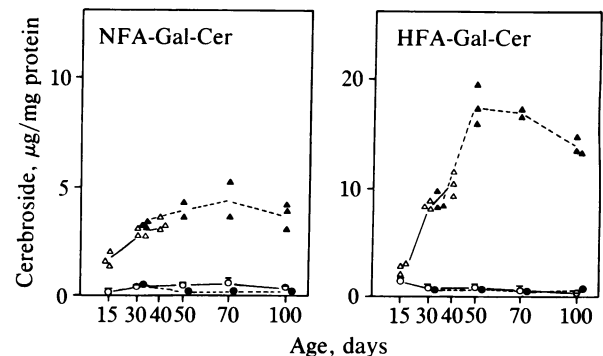


FIG. 6. Concentration of nonhydroxy- (Left) and hydroxy- (Right) cerebroside in kidneys of twitcher and control mice as a function of age. See legend to Fig. 1 for the symbols.

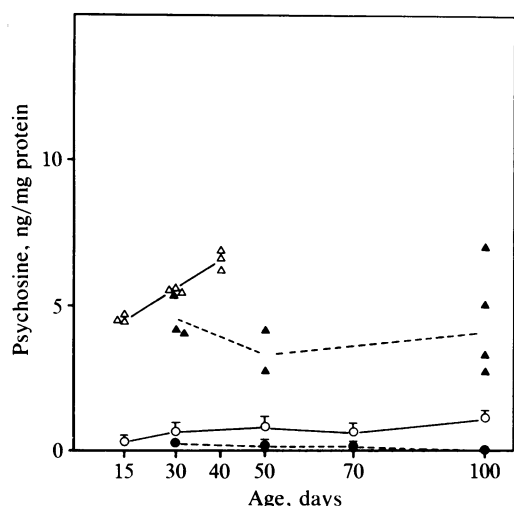


FIG. 7. Concentration of psychosine in kidneys of twitcher and control mice as a function of age. See legend to Fig. 1 for the symbols.

HCT were similar to those in preterminal (40-day-old) untreated animals. In addition, cerebroside levels in kidney also appeared to be unaffected by HCT in either twitcher or control mice (Fig. 6). Psychosine levels in the kidney of transplanted twitchers were also unchanged in 30-day-old mice, decreased until animals reached 50 days of age, and then leveled off (Fig. 7).

## DISCUSSION

Although the enzymatic defect is the same in human Krabbe disease patients and twitcher mice, there are several differences in the clinicopathological manifestations. While Krabbe disease affects primarily the central nervous system, the twitcher mutation affects primarily the peripheral nervous system. In Krabbe disease, severe myelin defects occur in the brain and result in significantly lower content of cerebroside, despite a lack of galactosylceramidase (galactocerebroside). In contrast, only a slight decrease in cerebroside has been found in the brains of twitcher mice near death (7), an observation confirmed by our present study. Unlike the relatively mild involvement of brain, the peripheral nerves of twitcher mutants suffer a severe myelin defect (8, 9). To confirm this observation, and in agreement with the study by Igisu and Suzuki (11), we found that the levels of cerebroside were significantly decreased in the sciatic nerves of twitchers. Besides the nervous system, the twitcher mouse kidney is known to contain high concentrations of cerebroside (12, 20). Our study confirmed these

observations and agree with the finding of abnormal inclusions in the twitcher kidney (10). It is not known whether elevated levels of these compounds contribute to renal dysfunction in twitcher mice.

As described previously, psychosine accumulates in the tissues of patients with Krabbe disease and in the twitcher mouse, and its toxic effects on neural tissues may be the major pathogenetic process in these conditions. In support of this hypothesis, Svennerholm *et al.* (3) and Igisu and Suzuki (5) found elevated psychosine levels in the brains of patients with Krabbe disease and the mouse mutants, respectively. In the present investigation, we also found highly elevated psychosine levels in the brains of twitcher mice. More importantly, we have demonstrated that extremely high concentrations of psychosine are also present in the sciatic nerves of the mutant animals. Psychosine levels (per mg of protein) in sciatic nerves were 5- and 7-fold higher than the levels in brains of 30- and 40-day-old twitchers, respectively. In fact, the levels of psychosine found in tissues of 40-day-old untreated twitcher mice (over 500 ng per mg protein) are well beyond the values observed in the brains of patients with Krabbe disease (3) and also exceed the concentration that is toxic to animal cells *in vitro* (21). Furthermore, although the absolute amount of psychosine in the twitcher kidney is low, its level is also significantly elevated beyond that observed in control normal littermates.

Twitcher mice usually die at 40 to 45 days of age even with optimal supportive care. Although the precise cause of death is not known, failure of the peripheral nervous system is suspected. The life span of the twitcher mouse is prolonged substantially by HCT (13). Although the present study demonstrated that HCT led to the appearance of galactosylceramidase (galactocerebroside) in twitcher tissues, including brain, there was no significant effect on the levels of cerebroside compared with the levels in age-matched untreated twitchers. The cerebroside content in brain and sciatic nerve was relatively unchanged until the HCT-treated animals reached 100 days of age; indeed, even with HCT, twitcher mice do not survive beyond 155 to 180 days of age (ref. 22; A.M.Y., unpublished observations). Despite modest increases in enzyme activity in the twitcher kidney, cerebroside levels in that organ continued to increase substantially until the animals reached 50 days of age, after which time cerebroside levels stabilized. In spite of relatively steady levels of cerebroside in the sciatic nerves of twitcher mice after HCT, the levels of psychosine gradually increased with age and reached the same level as in preterminal 40-day-old untreated animals (about 500 ng per mg protein) by the time that HCT-treated twitchers were 100 days of age. On the other hand, psychosine levels in brain and kidney were relatively unchanged during this period.

Table 1. Galactosylceramidase activity after HCT

Mice	Age at sacrifice, days	No. of animals	Galactosylceramidase activity, nmol/hr per mg protein			
			Tail tip at 7 days	Brain	Kidney	Spleen
<b>Twitcher</b>						
Non-HCT	40	10	0.034 ± 0.002	0.0186 ± 0.007	0.0176 ± 0.005	0.002 ± 0.0001
HCT	50	4	0.09 ± 0.07	0.075 ± 0.018	0.023 ± 0.012	0.21 ± 0.07
	70	4	0.05 ± 0.05	0.090 ± 0.061	0.146 ± 0.083	0.31 ± 0.01
	100	4	0.06 ± 0.02	0.341 ± 0.173	0.30 ± 0.10	1.90 ± 1.25
<b>Control</b>						
Non-HCT	40	10	0.74 ± 0.047	0.223 ± 0.04	1.005 ± 0.117	0.70 ± 0.02
HCT	50	3	0.79 ± 0.07	0.28 ± 0.10	0.34 ± 0.06	0.69 ± 0.15
	70	3	0.98 ± 0.08	0.59 ± 0.48	1.20 ± 0.12	0.74 ± 0.07
	100	5	0.87 ± 0.05	0.39 ± 0.18	1.62 ± 0.65	1.17 ± 0.02

Animals received HCT at age 10 days. Mean values ± SEM are shown.

The attainment of normal levels of galactosylceramidase in the brains of twitcher mice after HCT is an instructive observation, since it demonstrates that transfer of donor-derived enzyme takes place between the peripheral circulation and the central nervous system (CNS) in this animal model of a sphingolipid storage disease. Studies in the bovine model of mannosidosis indicate that hematopoietic chimerism with enzymatically normal donor cells favorably affects the visceral manifestations of this storage disease but fails to alter the pathology in the CNS, suggesting that circulating donor mannosidase did not cross the blood-brain barrier (23). Two clinical studies have reported that stabilization of CNS function may occur after allogeneic bone marrow transplantation (BMT) for human metachromatic leukodystrophy (arylsulfatase A deficiency), another sphingolipid storage disease (24, 25); furthermore, donor leukocytes were identified in the cerebrospinal fluid (CSF) from one of these patients after BMT, suggesting that donor-derived lymphohematopoietic cells had crossed the blood-brain barrier (24). Serial examinations of CSF from two patients who received allogeneic BMT for mucopolysaccharidosis I (the Hurler syndrome) have demonstrated a progressive decrease in the high levels of substrate (glycosaminoglycans) in the CSF after BMT, again indicative of some correction of the metabolic defect in the CNS by provision of enzyme from marrow-derived donor cells (26). It is not known whether the appearance of galactosylceramidase and stabilization of psychosine levels in the brains of twitcher mice after HCT are due to entry of enzyme or of donor cells into the CNS. Our findings and these clinical observations demonstrate that favorable biochemical alterations in the CNS may occur after BMT and that enzymic activity appears in the CNS even without specific strategies to perturb the blood-brain barrier, in contrast to the negative observations in bovine mannosidosis chimeras (23).

The precise mechanisms by which HCT prolongs survival in twitcher mice are unknown. In the present study, we found that the level of cerebroside was relatively unchanged by HCT. Since cerebroside is uniquely concentrated in myelin (27), this observation may indicate that no significant further demyelination occurs during the period of life extension and that progressive deterioration of the nervous system may not be the direct cause of death in transplanted twitcher mice. On the other hand, the levels of psychosine (the major toxic metabolite in this sphingolipid storage disease) were relatively low in the sciatic nerves of 50-day-old HCT-treated twitchers but in 100-day-old transplanted animals had reached the same high values observed in the nerves of 40-day-old untreated animals. The psychosine levels in both brains and kidneys of HCT-treated twitchers nevertheless remained stable during this period of observation. Although these findings might support the hypothesis that extremely high levels of psychosine cause death in transplanted twitchers by toxic effects on the peripheral nervous system, other studies suggest repair and preservation of function in peripheral nerves of twitcher mice after HCT. Light-microscopic examination of sciatic nerves in HCT-treated twitcher mice indicates some remyelination by 70–80 days after HCT (13, 22), and serial studies of sciatic motor nerve conduction velocities in transplanted twitchers demonstrate stabilization at subnormal values that allow neuromuscular transmission and preservation of motor function (28). Possibly, damage to oligodendroglia in the CNS and Schwann cells in the peripheral nervous system is advanced by the time that galactosylceramidase reaches the nervous system after 10-day-old

twitchers receive HCT. Studies of the survival, neuropathology, and biochemical parameters in twitcher mice that undergo HCT at age 5 days may provide an answer to this question.

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