

GENE THERAPY

Intravenous gene therapy improves life span and clinical outcomes in a feline model of Sandhoff disease

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Sandhoff disease (SD) is a fatal neurodegenerative disorder caused by the absence of β -N-acetylhexosaminidase (Hex) and subsequent accumulation of GM2 ganglioside in lysosomes. Previous studies have led to the development of an adeno-associated virus (AAV) vector-delivered gene therapy for children with GM2 gangliosidosis in both expanded access and phase 1/2 clinical trials through intrathalamic and cerebrospinal fluid-based delivery. This study investigated intravenous delivery of a bicistronic AAV vector-based gene therapy that has not yet been tested in clinical trials to a feline model of SD, treated presymptotically at 1 month of age. Whereas untreated SD cats lived to 4.3 ± 0.2 months, SD cats treated with low or high doses of the gene therapy lived to 8.3 ± 1.2 or 12.4 ± 2.7 months, respectively. In-life assessments revealed a clinical benefit of AAV treatment, with marked improvements seen in the prevention of overt full-body tremors; cerebrospinal fluid and serum markers of central nervous system damage were also reduced. Magnetic resonance imaging and spectroscopy indicated that the structural pathology and metabolite abnormalities seen in untreated SD cats were partially normalized by treatment. Ultrasound elastography showed improvement in the livers of cats in the high-dose treatment group. Dose-dependent reductions of GM2 ganglioside storage and increases in Hex activity were documented, associated with reduced neuroinflammatory cell populations and partial correction of myelin deficits. These data support the dose-dependent efficacy of intravenous-delivered gene therapy for restoration of Hex activity and preservation of clinical metrics, supporting potential for translation to patients with SD.

INTRODUCTION

Sandhoff disease (SD) and Tay-Sachs disease (TSD) are neurodegenerative lysosomal storage diseases that result in the death of affected children by around 4 years of age. Because there are no US Food and Drug Administration (FDA)-approved therapies, current treatment strategies are limited to palliation. SD and TSD are clinically similar forms of GM2 gangliosidosis (GM2), and their pathogenesis results from the absence of β -N-acetylhexosaminidase (Hex) and subsequent accumulation of GM2 ganglioside in lysosomes. Hex consists of two subunits, α and β , encoded by the *HEXA* and *HEXB* genes, respectively. The subunits dimerize to form distinct isozymes: HexA ($\alpha\beta$) is solely responsible for GM2 ganglioside degradation in humans, whereas HexB ($\beta\beta$) catabolizes neutral substrates including glycosaminoglycans. Distinct forms of GM2 disease are defined by deficiency of the α (TSD) or β (SD) subunit, yet the clinical phenotype is similar in both disorders (1). The deficiency of Hex activity results in a cascade of pathological effects throughout the body, with the most severe dysfunction in the central nervous system (CNS).

Although enzyme replacement, stem cell, and chaperone therapies (2) continue to be explored as treatment strategies for SD and TSD, none have yet achieved the level of efficacy in animal models

that gene therapy has. Through injection of adeno-associated virus (AAV) vectors expressing cDNAs for both the α and β subunits, Hex activity and GM2 ganglioside storage deficits have been consistently improved via a number of delivery routes and in different animal models (3–8). AAV1, AAV2, and AAVrh8 were used for direct injections of the brain in early investigations, with treatment consisting of both subunits of Hex (α and β) expressed from separate vectors in a 1:1 mixture (5, 9). Bilateral injection of AAVs into the thalami and deep cerebellar nuclei (DCNs) of affected SD cats quadrupled life span and completely abrogated full-body tremors, the most debilitating feature of feline SD. AAV injection of the CNS was so effective that about half of the treated SD cats died of emergent peripheral organ disease, which remained subclinical in untreated cats because of their severe and rapidly progressive CNS dysfunction (10).

Concerns about injection into the highly vascular posterior fossa, where the cerebellum resides, led to the investigation of less invasive routes. Several reports have suggested that cerebrospinal fluid (CSF) is an effective substitute for DCN injection in mice, cats, and sheep (8, 11, 12). AAV delivery via CSF was refined in sheep by using a flexible catheter inserted at the lumbar spinal cord and advanced rostrally into the cisterna magna (13). The studies described above led to expanded access (14) or phase 1/2 clinical trials in children with GM2 gangliosidosis using AAV vectors expressing the Hex α and β subunits delivered to the thalamus and CSF or to the CSF alone (ClinicalTrials.gov, identifiers NCT04669535 and NCT04798235).

Intravenous delivery of gene therapy is a less invasive approach that may effectively treat the CNS when using a capsid that can penetrate the blood-brain barrier (BBB), such as AAV9 (15). Although debate continues about the pros and cons of intravenous versus CSF-based treatment approaches for CNS disorders (16), numerous reports in animal models have demonstrated the efficacy of AAV-based gene

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therapies delivered by the intravenous route (7, 17–22), and intravenous-delivered AAV9 has been approved by the FDA and the European Medicines Agency for treatment of spinal muscular atrophy (23). Positive results of a phase 1/2 clinical trial of AAV9 delivered intravenously to children with GM1 gangliosidosis (NCT03952637) further support the potential of this approach. However, simultaneous expression of both the α and β subunits of Hex is essential for optimal production of HexA (24–26), the isozyme responsible for GM2 ganglioside degradation in humans. Delivery of two separate vectors, each expressing a Hex subunit, is not a viable strategy for intravenous injection because of vector dilution in the bloodstream and low likelihood that cells will be transduced by both vectors. Thus, the purpose of this study was to test a bicistronic vector expressing both Hex α and β subunits (20) in the feline model of SD for potential translation to human patients. If effective, the current bicistronic vector design, which has not been tested in patients to date, will be further developed for a future clinical trial to treat both SD and TSD.

RESULTS

Intravenous gene therapy improves life span and quality of life

SD cats were treated between 0.77 and 1.47 months of age (mean, 1.10 ± 0.15) by intravenous injection of a bicistronic AAV9 vector expressing both the α and β subunits of Hex as previously described (20), except that feline-specific cDNAs were used (see Materials and Methods). The bicistronic vector consisted of a centrally located cytomegalovirus (CMV) enhancer flanked on either side by chicken β -actin promoters in opposite orientations. Complementary DNAs for the feline Hex α and β subunits flanked the central regulatory elements and were followed by a SV40 or rabbit β -globin polyadenylation signal, respectively. The vector was designated AAV9-Bic-fHex. Although

clinical symptoms were absent at this time point, neuropathology develops in utero in humans with TSD and SD (27–31). A single injection of vector at 5×10^{13} vector genomes (vg)/kg (low dose) or 2×10^{14} vg/kg (high dose) was administered through the cephalic vein over ~2 min. No sedation was required. No pretreatment or immunosuppressive agents were administered.

The life spans of SD cats were almost doubled or tripled by AAV treatment with the low or high dose, respectively (untreated, 4.3 ± 0.2 months; low dose, 8.3 ± 1.2 months; and high dose, 12.4 ± 2.7 months). The longest-lived cat (ID: 55) was 16.0 months old at humane end point, defined as the point at which the cat could no longer stand, usually because of pelvic limb weakness or paresis (Fig. 1A). Moreover, AAV treatment improved body weights (Fig. 1B) and neurological clinical rating scores (Fig. 1C) in a dose-dependent fashion in treated SD cats, although even the high dose failed to normalize both metrics. The most marked clinical improvement was seen in the prevention of body tremors, the most debilitating feature of feline SD. Whereas untreated SD cats developed overt full-body tremors by 2.4 ± 0.1 months, SD cats treated with the high dose never developed full-body tremors, and, in the low-dose cohort, one of the four cats did not develop full-body tremors, whereas the remaining three cats had a delayed onset at 6.8 ± 1.8 months of age ($P = 0.034$) (Table 1). Cats in the high-dose, long-term follow-up cohort reached humane end point through pelvic limb paresis (Table 1) because of spinal cord compression (fig. S1), instead of debilitating full-body tremors as seen in untreated SD cats.

Ultrasound elastography demonstrates transient efficacy in liver

Ultrasound elastography measures the elastic properties of soft tissue through the propagation of shear waves. A loss of elasticity or increase in tissue stiffness is reported in common conditions such as

Fig. 1. Survival and quality of life improve with intravenous administration of AAV. (A) Kaplan-Meier curves. Mantel-Cox test. * $P < 0.05$ versus untreated SD cats; + $P < 0.05$ versus low dose. (B) Weights of treated cats. Error bars represent means $\pm$ standard deviation. Ordinary one-way ANOVA with Fisher's post hoc comparisons only for untreated versus all groups and low dose versus high dose. * $P < 0.05$ normal versus untreated SD cats. (C) Neurological clinical rating scores.

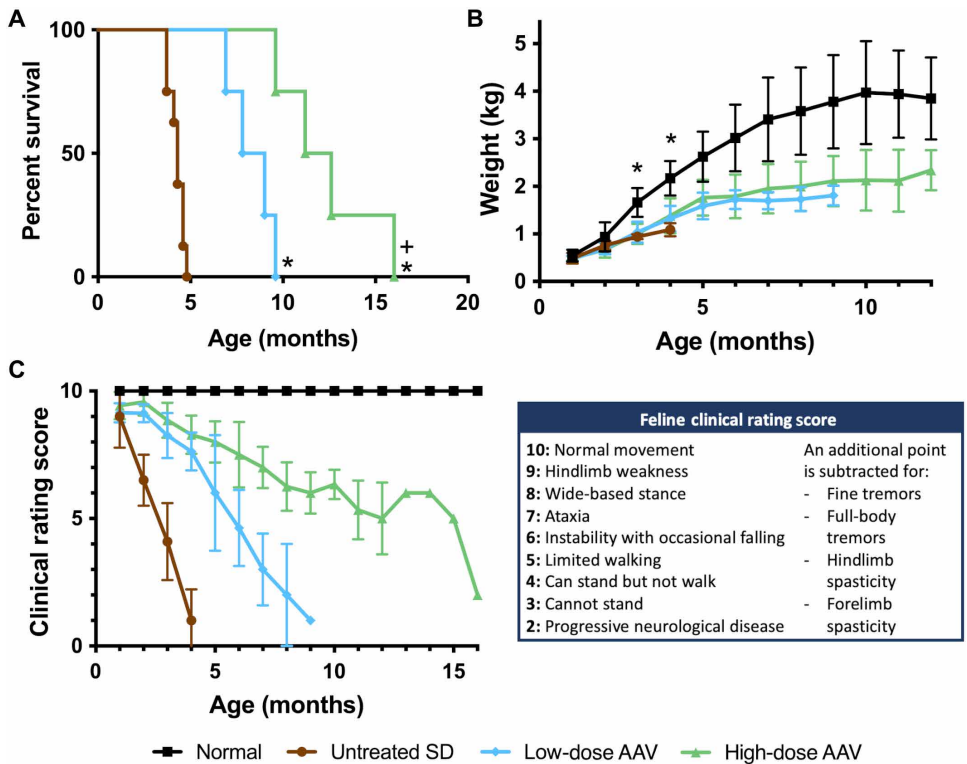


Table 1. AAV-treated SD cats and their clinical outcomes. CRS, clinical rating score.

Dose (vg/kg)	Follow-up	Cat ID	Survival (months)	Final CRS	Overt tremor onset (months)	Reason(s) for humane end point
Low (5 × 10 ¹³)	Short	26	*	3	None	*
		25	*	4	None	*
		57	*	8	None	*
		93	*	9	None	*
	Long	09	9.0	1	4.9	Overt full-body tremors
		17	9.6	1	8.5	Overt full-body tremors
		20	7.8	0	7.0	Overt full-body tremors
		27	6.9	1	None	Pelvic limb paresis
	Short	16	*	8	None	*
		11	*	8	None	*
96		*	9	None	*	
High (2 × 10 ¹⁴)	Long	29	12.6	4	None	Pelvic limb paresis
		53	11.2	6	None	Pelvic limb paresis or bilateral grade 4/4 luxating patellae
	Long	52	9.6	5	None	Pelvic limb paresis
		55	16.0	2	None	Pelvic limb paresis/rapid disease progression

*Predetermined experimental end point for cats in the short-term cohort was 16 weeks posttreatment, at which time they had not reached humane end point; long-term animals were followed to humane end point on the basis of neurologic function.

breast cancer (32) and liver fibrosis (33). We hypothesized that an increase in storage material in SD tissues would decrease elasticity (or increase stiffness), and we therefore included elastography as an exploratory outcome measure because of its low invasiveness and potential to track peripheral disease.

Two common readouts from elastography are wave speed and elasticity, which, in the livers and spleens of SD cats, were significantly reduced compared with normal (Fig. 2) (liver speed, $P = 0.0003$; elasticity, $P = 0.0006$; spleen speed, $P = 0.0029$; elasticity, $P = 0.0041$). AAV treatment led to a transient, dose-dependent pattern of improvement in the liver. Wave speed and elasticity approached normal in the livers of SD cats treated with the high dose until 7 months of age but returned to untreated levels at later time points, whereas SD cats treated with the low dose had wave speed and elasticity reductions similar to those in untreated SD cats at all time points (Fig. 2A). Untreated SD and AAV-treated cats showed no meaningful change in elastography metrics in the kidney, pancreas, or skeletal muscle (fig. S2).

MR images (7 T) and MRS metabolites support partial vector efficacy in the brain

We next assessed T2-weighted magnetic resonance (MR) images acquired with a 7-T scanner of normal and SD cats, both untreated and treated (Fig. 3A). In T2 images from normal cats, white matter was darker than (hypointense to) gray matter in the cerebrums and cerebellums. The corona radiata could be differentiated from surrounding gray matter of the parietal cortex, and the white matter of the DCNs was distinct from the overlying cerebellar cortex. In untreated SD cats, an increase in white matter signal intensity (because lipid-rich myelin was depleted) and decrease in gray matter signal intensity (because lipid-rich storage material was increased) caused

the normally clear gray-white matter boundaries to become isointense (34–36). In addition, the hyperintense CSF signal surrounding the brain and gyri became wider in SD cats as the brain parenchyma atrophied and CSF filled the resulting space. Images of AAV-treated cats showed partial restoration of the gray/white matter boundaries in the cerebrum and cerebellum, especially in the high-dose, short-term cohort imaged 16 weeks after treatment. Similarly, the most pronounced preservation of CSF volumes was apparent in the high-dose, short-term cohort. As AAV-treated cats in the low-dose and high-dose cohorts neared humane end point, deterioration of brain magnetic resonance imaging (MRI) architecture became more similar to that of untreated SD cats again (Fig. 3A). Thus, MRI architecture of the brain correlated well with clinical disease in AAV-treated and untreated SD cats over time.

MR spectroscopy (MRS) data suggest that the cerebellums were more affected in untreated SD cats than the parietal cortices or thalami, with significant increases in both *N*-acetylaspartate and *N*-acetylhexosamine (NAA + NA-Hex) ($P < 0.0001$) and glycerophosphocholine and phosphocholine (GPC + PCh) ($P = 0.0354$) (Fig. 3B). Whereas NAA reflects neuronal health and is decreased in many neurodegenerative storage diseases, NA-Hex is a storage product that cannot be resolved spectroscopically from NAA. Thus, the overlapping spectra of NAA + NA-Hex serve as a biomarker of storage in the feline SD brain, with elevations also occurring in the parietal cortex and thalamus (37, 38). In contrast, the parietal cortices and thalami of untreated SD cats had normal amounts of GPC + PCh, an indicator of membrane turnover that often correlates with demyelination. Myoinositol (Ins), thought to be an indicator of neuroinflammation, remained unchanged in all three brain regions (Fig. 3B).

The high-dose AAV treatment normalized NAA + NA-Hex in all three brain voxels, in both short-term and long-term cohorts. A

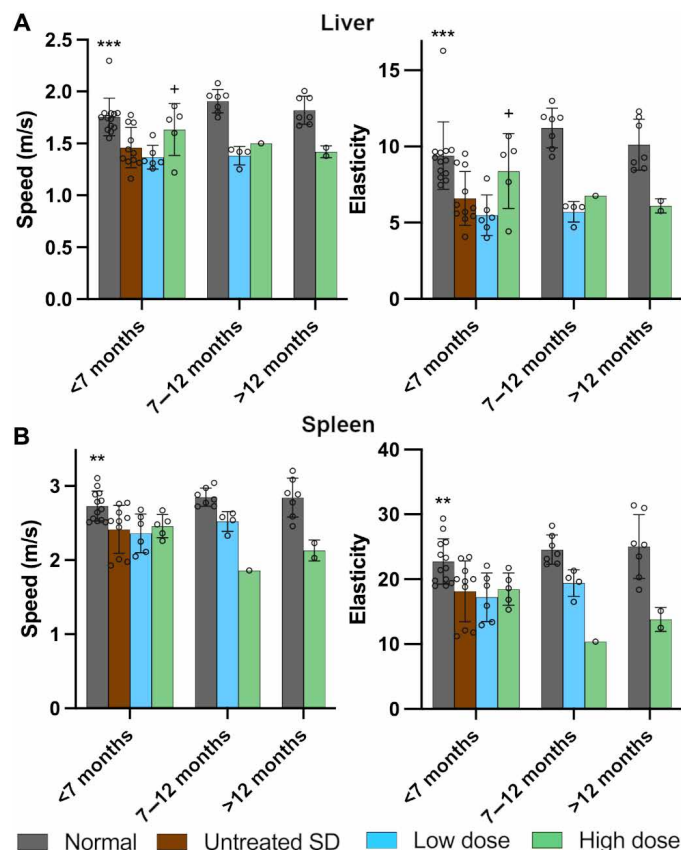


Fig. 2. Liver stiffness in SD cats is improved with high-dose AAV treatment. (A and B) Shear wave ultrasound elastography of liver (A) and spleen (B). No data are available for untreated SD cats at 7 to 12 months and >12 months or for the low-dose cohort at >12 months because animals did not survive to those time points. Data were collected at 7 to 12 months for only one cat in the high-dose cohort. Error bars represent means $\pm$ standard deviation. Ordinary one-way ANOVA with Fisher's post hoc comparisons only for untreated versus all groups and low dose versus high dose. ** $0.01 > P > 0.001$ and *** $P < 0.001$ compared with untreated SD cats; + $0.05 > P > 0.01$ versus low dose.

dose effect was noted in the cerebellums and thalami of SD cats, with the low dose yielding NAA + NA-Hex levels that were intermediate between the untreated and high-dose cohorts. Cats treated with the high dose of AAV had normal levels of GPC + PCh in the cerebellums, although levels in the parietal cortices and thalami were not different from those in untreated SD cats (Fig. 3B).

Body fluid biomarkers of pathology are improved after intravenous treatment

CSF concentrations of aspartate aminotransferase (AST) and lactate dehydrogenase (LDH), indicators of cell damage in the CNS, were significantly increased in untreated SD cats at humane end point (4 to 5 months of age) (Fig. 4, A and B) ($P < 0.0001$). AAV-treated SD cats had less AST and LDH at 5 months of age than untreated SD cats, with the high dose being more effective at reducing these concentrations (Fig. 4, A and B). Specific activity (SA) of all isozymes of Hex (total Hex) was decreased to background levels (6.85% of normal) in untreated SD cats and restored to near- or above-normal levels in cats treated with both doses across ages (Fig. 4C). Also, serum levels

of neurofilament light chain (NfL) were threefold above normal in untreated SD cats and corrected to near-normal amounts in all treatment groups (Fig. 4D).

Peripheral organ pathology is improved by intravenous treatment

Peripheral organ pathology previously reported in SD cats treated by brain injection of monocistronic vectors (10) was mild in the bicistronic intravenous-treated cats of the current study. Although one intravenous-treated animal of the long-term cohorts was judged to have stomach enlargement in life, none had an enlarged stomach at necropsy. Similarly, no animals had an enlarged colon at necropsy. Bladder volumes in intravenous-treated SD cats (low dose, 10.9 ± 5.0 ml/kg; and high dose, 14.0 ± 5.5 ml/kg) were increased compared with those in normal cats (2.3 ± 1.1 ml/kg body weight) and untreated SD cats (5.5 ± 4.2 ml/kg). However, bladder volumes in intravenous-treated cohorts were approximately half those of cats treated in the thalami and DCNs in our previous study (24.0 ± 9.3 ml/kg) (10). Mild to moderate patellar luxation in the long-term cohorts was noted in two of the four cats treated with the low dose and three of the four cats treated with the high dose. Patellar luxation, also common among cats treated by intraparenchymal injection, was considered to be secondary to hindlimb muscle weakness typical of feline SD. No animal in this study (treated or untreated) showed evidence of hepatocellular carcinoma, which has been reported to result from AAV treatment in some instances (39, 40).

Intravenous gene therapy increases HexA activity and decreases GM2 ganglioside abundance in the CNS

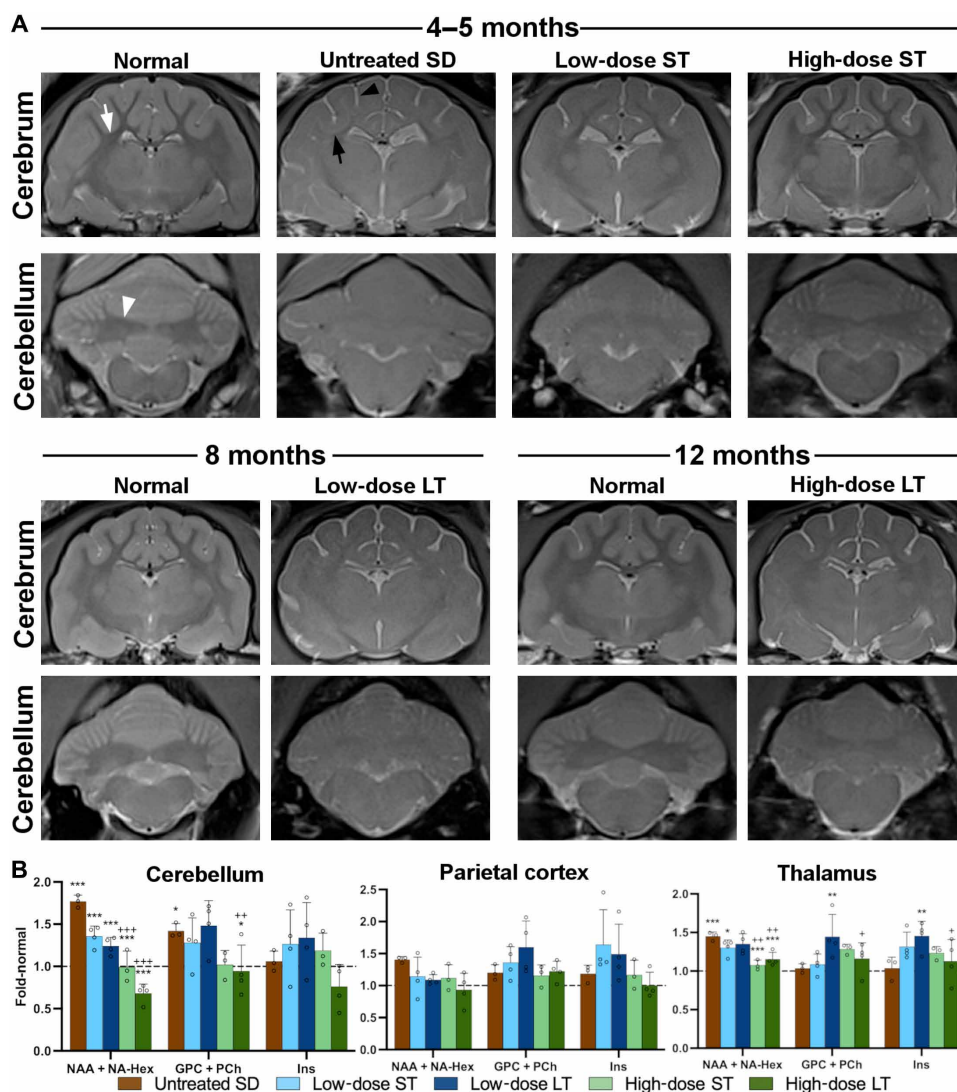
SA of HexA, the isozyme responsible for degrading GM2 ganglioside, was improved in AAV-treated cohorts, with variability dependent on CNS region and dose. In general, HexA activity remained near baseline (untreated) levels in the rostral portion of the forebrain, with a slight, dose-dependent increase in the caudal forebrain (Fig. 5A, sections A to F; section designations shown in Fig. 5B). In the hind-brains and spinal cords of high-dose treated SD cats, HexA activity increased above untreated SD levels in places, ranging from 0.7- and 1.2-fold normal, respectively (Fig. 5A, sections G to P; $P < 0.05$). A dosage effect was noted in the hindbrains and spinal cords, where mean HexA activity in the high-dose cohort was 6.13- and 6.73-fold that of the low-dose cohort, respectively. Based on restoration of HexA activity, the spinal cords of the high-dose cohort were treated most effectively of all CNS regions, with mean activity ranging from 0.2- to 1.0-fold normal HexA in the long-term cohort (Fig. 5A). Similar results were obtained when using a substrate that measures the activity of all Hex isozymes (MUG; fig. S3).

The activity of lysosomal enzymes other than Hex was above normal in untreated SD cats, a well-known phenomenon thought to represent a compensatory mechanism when one lysosomal enzyme is missing (9, 41). Lysosomal enzymes β -galactosidase and α -mannosidase had high levels of activity in untreated SD cats that were reduced toward normal in effectively treated regions of the brain and spinal cord (fig. S3).

GM2 ganglioside was elevated throughout the CNSs of untreated SD cats compared with normal cats, in which GM2 ganglioside was below the limit of detection (Fig. 5C). In SD cats treated with the high dose of AAV, mean levels of GM2 were reduced throughout the CNSs at 16 weeks posttreatment. At humane end point, GM2 levels had returned to those of untreated SD cats in the cerebrum but

Fig. 3. AAV treatment partially normalizes 7-T

MR images and MRS metabolites. (A) Representative T2-weighted anatomical images. In normal cats, white matter is darker than, or hypointense to, gray matter in the corona radiata of the cerebrum (white arrow) and the DCNs of the cerebellum (white arrowhead). The CSF signal (bright) around the meninges is minimal in normal cats. In untreated SD cats at humane end point, the DCNs are isointense to (same shade as) surrounding gray matter, and the coronae radiatae (black arrow) are hyperintense (lighter than) gray matter because of hypomyelination and storage in neuron cell bodies. The increased amount of CSF signal (black arrowhead) in untreated SD cats is especially apparent surrounding the gyri and is attributed to generalized atrophy. **(B)** MRS data for three brain voxels: cerebellum, parietal cortex, and thalamus. The spectroscopic peak of the neuro-metabolic marker NAA merges with the NA-Hex storage product in SD. ST, short term; LT, long term. Error bars represent means $\pm$ standard deviation. Ordinary one-way ANOVA with Fisher's post hoc comparisons only for untreated versus all groups and low dose versus high dose. * $0.05 > P > 0.01$, ** $0.01 > P > 0.001$, and *** $P < 0.001$ versus untreated SD cats (* over untreated SD bar indicates comparison with age-matched normal); ++ $0.01 > P > 0.001$ and +++ $P < 0.001$ versus low dose at same age. Dashed horizontal line represents levels in age-matched normal cats, to which all SD data were standardized.



remained lower in the cerebellum, brainstem, and spinal cord, corresponding to the higher HexA activity in those regions. GM2 levels in the CNSs of the low-dose cohort were more variable, with significant reduction in the thalami ($P = 0.0004$) and cerebellums ($P = 0.0006$) at 16 weeks posttreatment but no reduction in other regions. At humane end point of the low-dose cohort, GM2 again remained at untreated levels in most regions and was even significantly elevated in the parietal cortices ($P = 0.0204$), temporal lobes ($P = 0.0074$), and occipital cortices ($P = 0.0015$) (Fig. 5C). Levels of sialic acid, a constituent of GM2 ganglioside, generally followed the same pattern as those of GM2 in the CNSs of untreated and treated SD cats (fig. S4).

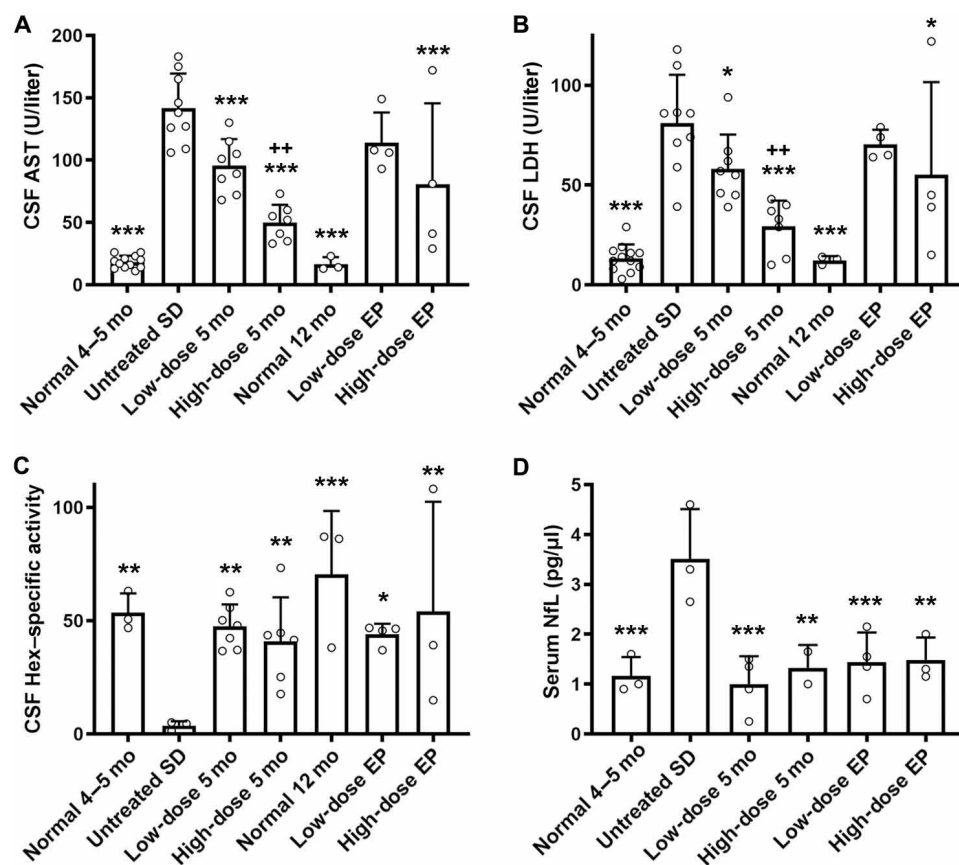
HexA activity in the CNS was plotted against survival in all animals followed to the humane end point. The region with the strongest correlation between HexA activity and survival was CNS block I, which includes the caudal cerebellum and brainstem [coefficient of determination (R^2) = 0.736; Fig. 5D]. Other brain regions with strong correlation between HexA activity and survival were brain blocks C (motor cortex, $R^2 = 0.501$) and E (thalamus, $R^2 = 0.652$) and cervical spinal cord blocks J and K ($R^2 = 0.558$ and 0.702 , respectively)

(Fig. 5E). Similar results were reported previously in cats treated by injection of the thalami and DCNs with monocistronic vectors (10).

Restoration of HexA activity varies in peripheral tissues

HexA activity in peripheral tissues of AAV-treated SD cats varied according to tissue type and duration of follow-up. Normal to above-normal HexA activity was measured in the heart, skeletal muscle, and adrenal gland, whereas only background activity was measured in the spleen and duodenum (Fig. 5F). Dose and treatment duration seemed to influence activity in some tissues like the heart, where HexA ranged from a low of 0.19-fold normal (low dose, short term) to a high of 0.84-fold normal (high dose, long term), and skeletal muscle, where activity was only 0.2- to 0.3-fold normal in short-term cohorts but increased to 1.1- to 2.1-fold normal in long-term cohorts, suggesting that HexA activity increased slowly over many months in these tissues. The strongest correlations between HexA activity and survival were measured in skeletal muscle ($R^2 = 0.708$) and liver ($R^2 = 0.577$), whereas heart and other tissues exhibited weak correlations (Fig. 5E). The production of both HexA and HexB isozymes was confirmed by ion exchange chromatography (Fig. 5G).

Fig. 4. AAV treatment reduces CSF and serum markers of cell damage and increases Hex activity. (A and B) CSF levels of AST (A) and LDH (B). **(C)** SA of all Hex isozymes (total Hex). **(D)** Serum levels of NFL. EP, humane end point of long-term cohort. Error bars represent means $\pm$ standard deviation. Ordinary one-way ANOVA with Fisher's post hoc comparisons only for untreated versus all groups and low dose versus high dose. * $0.05 > P > 0.01$, ** $0.01 > P > 0.001$, and *** $P < 0.001$ versus untreated SD cats; ++ $0.01 > P > 0.001$ versus low dose at same age.



In general, the highest levels of HexA activity were in tissues with the highest number of vg. However, an exception was noted in the liver, which had low HexA activity that never exceeded 0.09-fold normal at any dose or time point, although liver had the highest number of vg of all tissues (fig. S5). As a measure of expression efficiency, we calculated a SA:vg ratio across tissues. The higher the SA:vg ratio was, the more effective the production of HexA activity from a given number of vg. The SA:vg ratios in the brain, spinal cord, skeletal muscle, and heart ranged from 23.0 to 139.8 times higher than in the liver.

Neuroinflammation is reduced by intravenous gene therapy

Gliosis is a known feature of untreated SD cats as shown by abnormally high levels of the astrocytic marker glial fibrillary acidic protein (GFAP) and the microglial marker Iba1 (ionized calcium-binding adaptor molecule 1) throughout the brain. In most brain regions examined, mean GFAP immunostaining and cDNA copies were increased in untreated SD cats but only significantly increased in the parietal cortex gray matter ($P = 0.0199$) (Fig. 6A). Although increases in Iba1 immunostaining were less pronounced, microglial activation was distinct through changes from a ramified morphology, with a small cell body and long, branching processes in the normal cat brain, to an ameboid morphology, with an enlarged cell body and shortened, blunt processes in the SD cat brain (Fig. 6B).

With AAV treatment, GFAP staining, cDNA abundance, and glial cell morphology were largely normalized in animals of the high-dose, short-term cohort, but inflammatory changes increased with age in the long-term cohorts. In general, AAV-treated cats in the short-term

follow-up cohorts that were euthanized 16 weeks posttreatment had values at or below untreated SD levels (Fig. 6A). Cats in the long-term follow-up cohorts had higher levels of GFAP than their short-term counterparts in the parietal cortices, thalami, and cerebellar cortices, with values well above those of normal and untreated SD cats. The DCNs and brainstem regions were an exception, with minimal differences between the long-term and short-term AAV groups of either dose. In microglia, ameboid morphology was prominent in untreated SD cats, but ramified microglia predominated in normal and AAV-treated SD cats in the short-term cohort (Fig. 6B). In contrast, animals of the high-dose, long-term cohort (followed to the humane end point of 12.4 ± 2.7 months of age) had astrocytic and microglial morphology that resembled that in untreated SD cats, suggesting that glial cell pathology was reversed only temporarily by AAV treatment, consistent with a previous study in which SD cats treated by intracranial delivery of AAV had transient normalization of glial cell pathology (42).

Intravenous gene therapy partially restores neuronal protein expression

Cells immunostained for the neuronal markers NeuN or calbindin were reduced in a region-specific manner in untreated SD cats, with the number of NeuN-positive cells at 75 to 80% of normal in the parietal cortices and thalami, 30% of normal in the DCNs, and around normal levels in the cerebellar cortices and brainstems (fig. S6). Compared with untreated SD cats, the short-term cohort (16 weeks posttreatment) showed increased numbers of NeuN-positive cells, often achieving near-normal levels, especially with the high dose. Longer-lived

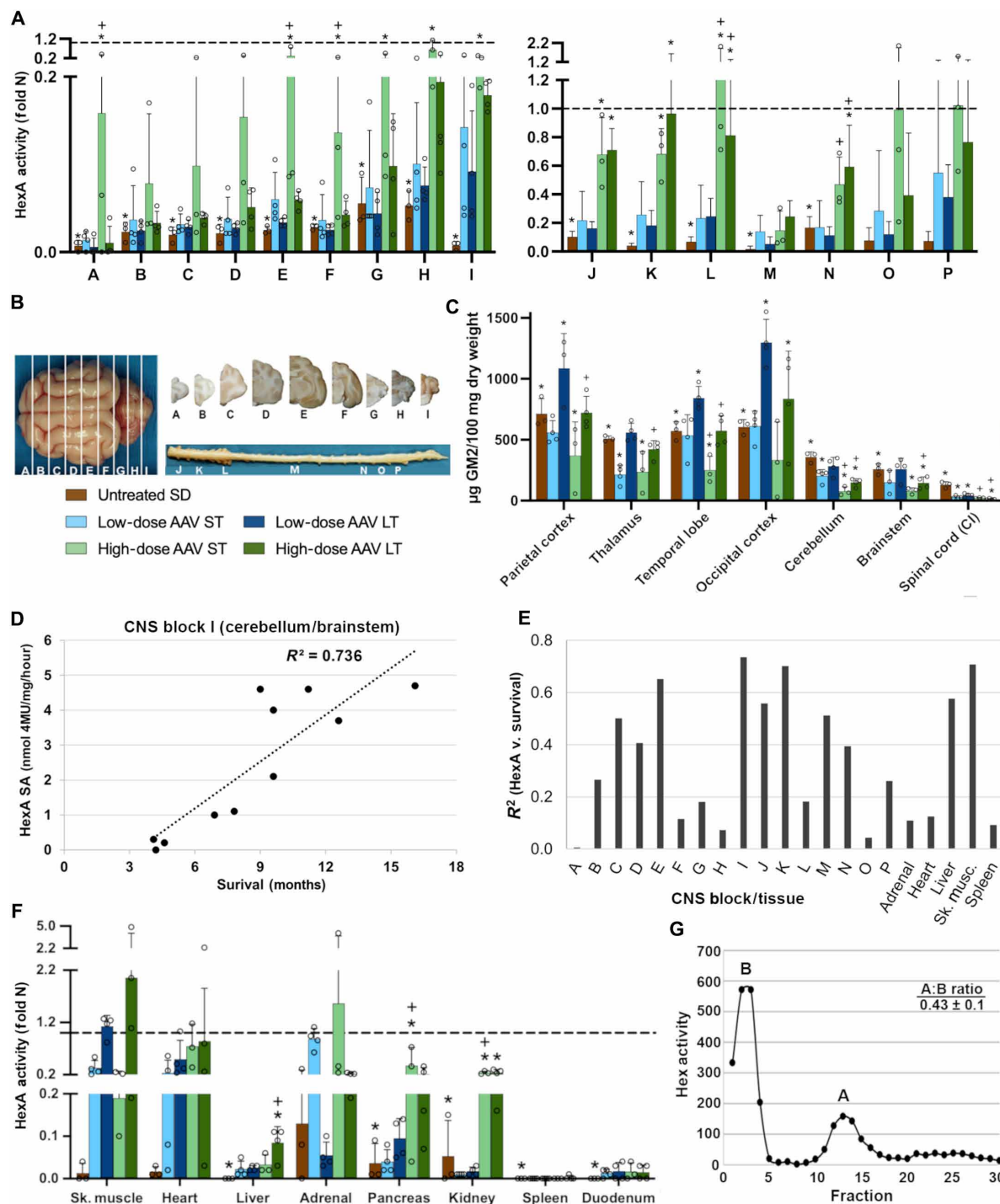


Fig. 5. Hex activity increases and GM2 ganglioside storage decreases with AAV treatment. (A) HexA activity [fold normal (fold N)] in the CNSs of treated cats. Activity in each block of the CNS was compared with the equivalent block in untreated normal controls. Dashed line represents activity in normal animals. (B) Letter designations of brain and spinal cord blocks (A to P). The dorsal surface of the CNS is shown on a blue background, with corresponding transverse hemisections of the brain on a white background. Also shown is the color legend for study groups. (C) GM2 ganglioside in the CNSs of untreated and AAV-treated SD cats. GM2 ganglioside in normal controls was below the limit of detection. (D) R^2 between HexA SA and survival (in months) for CNS block I, which contains the caudal cerebellum and brainstem. (E) Correlation between HexA activity and survival in other CNS and peripheral tissues. Letter designations for CNS tissue blocks are shown in (B). (F) HexA activity in peripheral tissues. Dashed line represents activity in normal animals. (G) Representative chromatogram of Hex isozymes from the liver of a high-dose treated cat. A, HexA; B, HexB. Each isozyme's total activity against the MUG substrate was used to determine the A:B ratio of 0.43 ± 0.1 ($n = 3$). The A:B ratio in normal cat liver is 0.56 ± 0.23 . Error bars represent means $\pm$ standard deviation. Ordinary one-way ANOVA with Fisher's post hoc comparisons only for untreated versus all groups and low dose versus high dose. * $P < 0.05$ versus untreated SD cats (* over untreated SD bar indicates comparison with age-matched normal); + $P < 0.05$ versus low dose at same age.

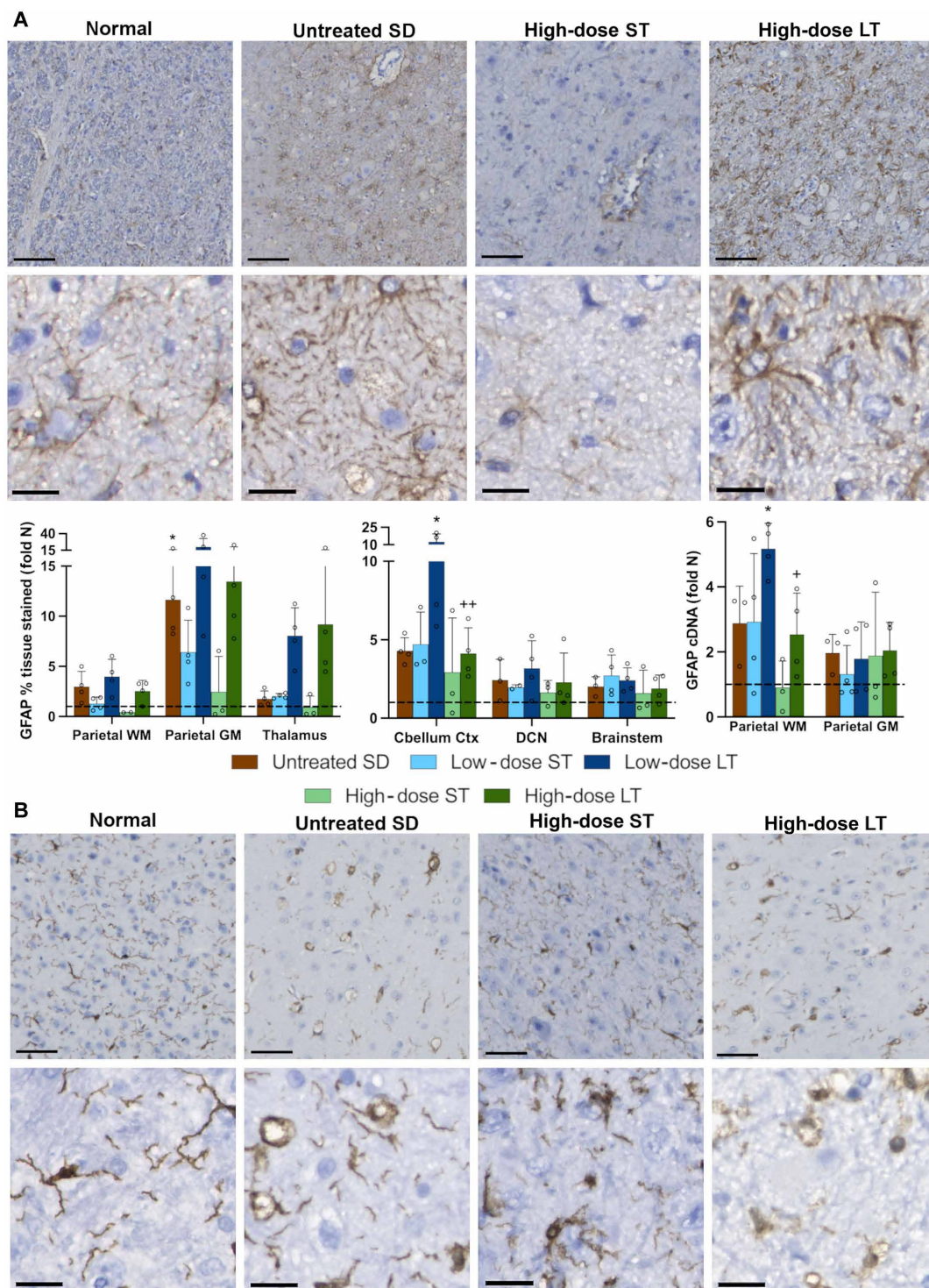


Fig. 6. Microglial activation is transiently prevented after AAV treatment. (A) GFAP immunostaining (brown) in representative sections from the thalamus. Graphs below the stained images quantify the percentage of tissue stained for GFAP in different regions of the CNS. Right-hand graph shows GFAP cDNA quantification by RT-qPCR in the parietal cortex. **(B)** Iba1 immunostaining (brown) in the parietal cortex. Each region of interest was drawn manually on a whole-slide image and then quantified in full. Scale bars: (A) 20 μ m (top) and 100 μ m (bottom), and (B) 20 μ m (top) and 50 μ m (bottom). WM, white matter; GM, gray matter; Cbellum Ctx, cerebellar cortex. Error bars represent means $\pm$ standard deviation. Ordinary one-way ANOVA with Fisher's post hoc comparisons only for untreated versus all groups and low dose versus high dose. $*0.05 > P > 0.01$ (* over untreated SD bar indicates comparison with age-matched normal); $+0.05 > P > 0.01$ and $++0.01 > P > 0.001$ versus low dose at same age. Dashed horizontal line represents levels in age-matched normal cats, to which all SD data were standardized.

treated cats had fewer NeuN-stained cells than their short-term counterparts, especially those administered the low dose, which showed lower mean values for NeuN immunostaining than untreated SD cats in all brain regions examined. Results were similar from stains for calbindin, a neuronal marker that includes Purkinje cells, in the cerebellar cortex (fig. S6).

Oligodendrocyte numbers and myelin staining are preserved in brain regions

The numbers of oligodendrocytes in the untreated SD cats, immunolabeled by Olig2, were reduced to around 50% normal in the parietal cortices (white matter, $P = 0.0001$; and gray matter, $P = 0.0002$) and thalami ($P < 0.0001$) (Fig. 7A). In the parietal cortex and thalamus, AAV treatment partially normalized the numbers of oligodendrocytes in a dose-dependent fashion. In the cerebellar cortices, DCNs, and brainstems, oligodendrocyte numbers were closer to normal with both dose groups, with long-term cats having above-normal levels of oligodendrocytes in the brainstems. Luxol Fast Blue staining indicated decreased myelin in untreated cats, which was restored in AAV-treated cats in a dose-dependent manner (Fig. 7B).

CNS histopathology is improved in intravenous-treated animals, especially in the short-term treatment groups

Histopathological changes for untreated SD cats at humane end point included moderate-to-marked neuronal swelling because of storage material, foamy macrophages surrounding vessels, mild-moderate gliosis, and dilated myelin sheaths with Gitter cells in white matter tracts throughout the CNS (fig. S7). Cats in the high-dose, short-term AAV cohort had the fewest lesions overall, with only mild, rare neuronal storage material and white matter deficits only evident in the cerebellum. The low-dose, short-term AAV cohort demonstrated some improvement in severity of CNS lesions, whereas the long-term cohorts of both doses had severe lesions to the level of untreated SD cats. In the liver, storage lesions (vacuolation) in hepatocytes were marked in untreated SD cats (fig. S7). Vacuolation remained apparent in most AAV-treated cohorts, consistent with the low HexA activity in the livers of treated cats. However, vacuoles appeared to be smaller in the high-dose cohort versus the low-dose cohort. The greatest improvement in hepatocyte pathology occurred in the high-dose, long-term cohort, which had the highest level of HexA activity.

AAV vector was distributed broadly and serum antibody titers to AAV9 were present in all treated animals

AAV vector was present throughout the CNSs and peripheral tissues in treated animals, with the high-dose and short-term groups generally having the highest concentrations (fig. S5). In the CNS, vector concentrations were highest in the cerebral cortex (0.050 to 9.658 vg per diploid genome), with the cerebellum, brainstem, and spinal cord having similarly low levels (0.004 to 1.02 vg per diploid genome). The concentrations of vector were more dependent on dose than time posttreatment, with the high-dose cohorts having more vector than low-dose cohorts in the CNS. Mean vector concentrations decreased from short-term to long-term cohorts in almost every CNS block, which could be due to vector loss in transduced, mitotic cells or to cell death related to an immune response or disease progression. Similar dose-dependent differences in vector copies were also noted in peripheral nervous system tissues (sciatic nerve, cervical and lumbar dorsal root ganglia), whereas other peripheral organs

were relatively homogeneous among cohorts. Among peripheral tissues, the liver had the highest level of vector. Tissues with vector <0.2 vg per diploid genome were kidney, spleen, duodenum, and pancreas. Posttreatment, all cats developed serum antibody titers to AAV9 ranging from 1:1000 to 1:256,000 (fig. S8). All cats for whom pretreatment serum was collected ($n = 13$) had undetectable serum titers below 1:8 dilution, the lowest dilution tested. Purified feline Hex is not available commercially or from other labs, so we were unable to test treated cats for humoral immunity to feline Hex.

DISCUSSION

Enzyme replacement as a treatment for lysosomal storage diseases has been contemplated since the 1960s to 1970s, when stored substrate was eliminated from mucopolysaccharidosis (MPS) fibroblasts by “corrective factors” from cell culture supernatant, cell homogenates, or normal body fluids (43). For conditions such as Gaucher disease type I, with primary pathology outside of the CNS, enzyme replacement therapy has been quite successful. Enzyme replacement is a far greater challenge for the ~70% of storage disorders that are neuropathic, like TSD and SD, in which the BBB blocks functional enzyme from entering the parenchyma of the brain and spinal cord. Thus, effective CNS delivery mechanisms may be the next great advance in treating storage disorders, akin to the discovery of corrective factors in the 1960s.

Such is the hope for AAV gene therapy, in which the vector is both a means of enzyme replacement and a mechanism for delivery across the BBB. In this study, an intravenous-delivered bicistronic AAV9 vector improved the life spans of SD cats in a dose-dependent manner; longevity almost doubled with the low dose and almost tripled with the high dose. Quality-of-life measures also improved in treated cats, which maintained higher body weights and clinical rating scores than their untreated counterparts. Similar to previous studies of AAV treatment in SD cats (10), the most marked clinical improvement was the prevention of severe overt tremors. Overt tremors developed in only 3 of the 15 AAV-treated cats in this study, all in the low-dose cohort, with the age of onset delayed by 5 months compared with those in untreated SD cats.

Although survival in intravenous-treated cats was promising, reaching threefold longer than untreated controls, it did not equal the quadrupling of life span achieved by intracranial treatment (10). In that study, intracranial injection corrected CNS pathology so effectively that approximately half of treated SD cats died of peripheral organ disease (10). Enlargement of the stomach, urinary bladder, and colon was found frequently in long-term follow-up after intracranial treatment but was not an obvious factor in the demise of intravenous-treated cats. Thus, intravenous administration likely corrected, at least in part, some of the peripheral organ pathology that was treated ineffectively by intracranial injection. These results have implications for many neuropathic storage diseases, in which neurologic dysfunction is the primary cause of death but the enzyme deficiency occurs in all tissues: Optimal approaches to treat both the CNS and periphery should be considered, because progressive disease in either compartment may be fatal.

Further confirmation that intravenous gene therapy addressed peripheral organ pathology came from ultrasound shear wave elastography, which demonstrated that liver stiffness in SD cats was corrected by the high dose of vector. This noninvasive method is becoming increasingly accepted for the monitoring of conditions including

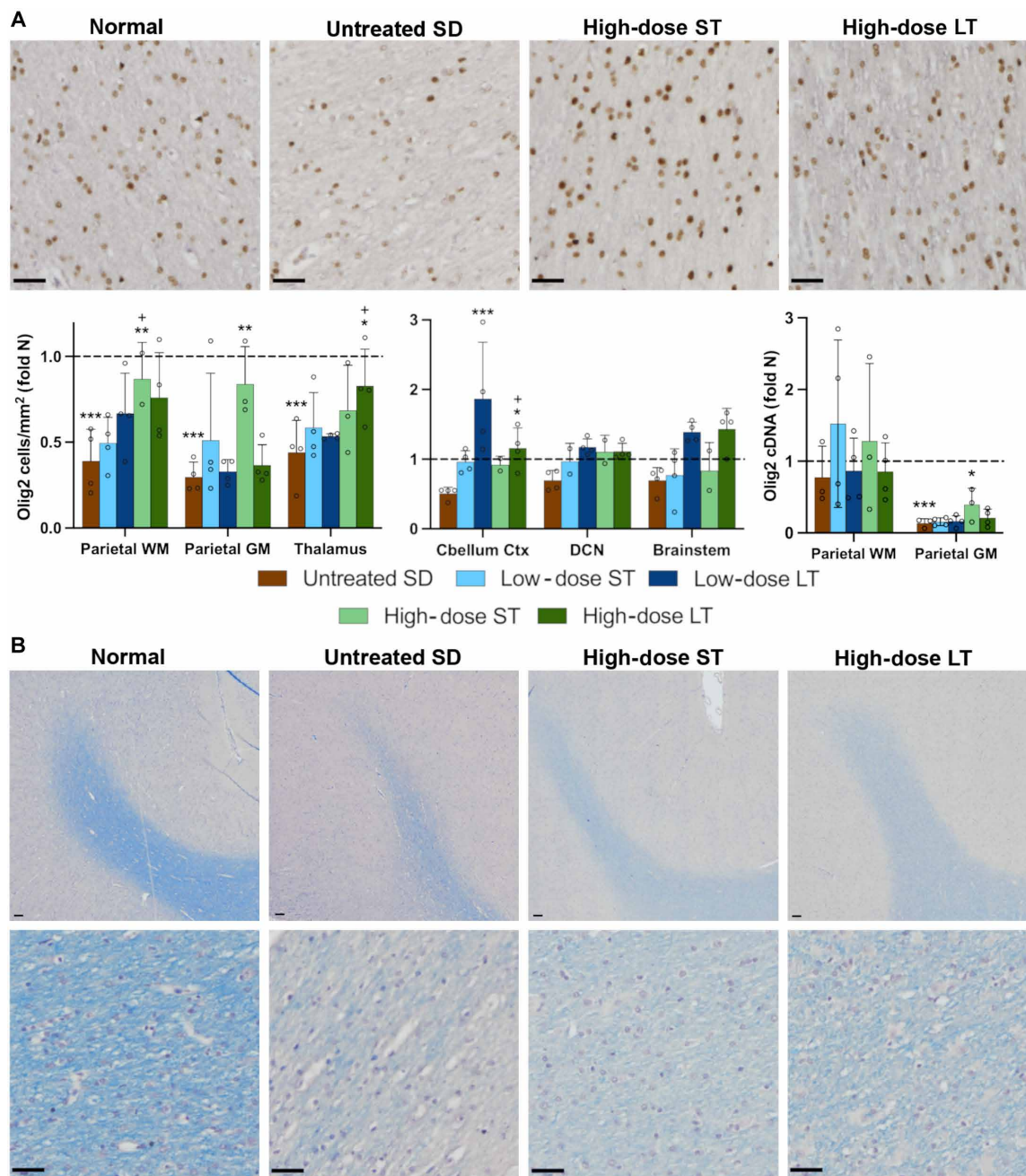


Fig. 7. Myelin deficits in SD cats are partially corrected through AAV treatment. (A) Olig2 immunostaining (brown) in representative sections from parietal cortex white matter. Graphs below the stained images quantify the percentages of tissue stained for Olig2 in different regions of the CNS. Right-hand graph shows Olig2 cDNA quantification by RT-qPCR in the parietal cortex. (B) Myelin stained by Luxol Fast Blue in the parietal cortices of representative cats. Each region of interest was drawn manually on a whole-slide image and then quantified in full. Scale bar: 20 μ m [(A) and (B), bottom] and 100 μ m [(B), top]. Ordinary one-way ANOVA with Fisher's post hoc comparisons only for untreated versus all groups and low dose versus high dose. * $0.05 > P > 0.01$, ** $0.01 > P > 0.001$, and *** $P < 0.001$ versus untreated SD cats (* over untreated SD bar indicates comparison with age-matched normal); + $0.05 > P > 0.01$ versus low dose at same age. Dashed horizontal line represents levels in age-matched normal cats, to which all SD data were standardized.

liver fibrosis (33), intervertebral disc pathology (44), and cardiac disease (45) in human patients and, recently, was adapted for use in feline clinical medicine (46–48). Elastography shows promise for monitoring peripheral organ disease in clinical trials.

Partial or temporary improvement of liver pathology can be explained by low HexA activity despite high vg in livers of AAV-treated

cats. Because liver has the highest HexA activity of almost all tissues in normal cats, a given SA expressed relative to normal will be lower in liver than in other tissues. Vector design also may play a role, in that our vector lacked the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) reported to increase AAV expression in mouse and rat livers. Although WPRE also increased

expression in muscle (2.7-fold), the magnitude of increase was greatest in liver (5.8-fold) (49). Last, cat liver may be a poor environment for gene expression from certain AAV designs. When HexA activity was standardized to vg in AAV-treated cats, liver produced the lowest activity per vg.

A peripheral disease manifestation ineffectively addressed by both intracranial (10) and intravenous routes was spinal cord compression, evident upon necropsy of most AAV-treated cats. Clinically, these cats displayed pelvic limb paresis or paralysis that led to the predetermined humane end point (inability to stand). Bony abnormalities have been recorded in radiographs and MR images in the cervical spine and coxofemoral regions in SD cats (50). Similar clinical signs are observed in human patients with MPS, a family of lysosomal storage diseases that primarily affects connective tissue (51). In animal models of MPS, spinal cord compression has been refractory to treatment, responding only to high-dose enzyme replacement at early ages (52). Future studies will need to focus on strategies to prevent or correct spinal cord compression, which will benefit patients with a variety of lysosomal storage disorders.

Salutary effects of intravenous gene therapy on astrogliosis were not shown in this study, but microglia maintained a resting morphology for 16 weeks posttreatment compared with an activated, amoeboid morphology in untreated SD cats. Similar short-term effects on microglia were shown in intracranially treated SD (42) and GM1 (53, 54) cats, but microglial activation was evident in longer-lived cats. It is possible that AAV treatment resolves some but not all causes of neuroinflammation, delaying but not preventing its onset.

Although myelin and oligodendrocyte deficits have previously been demonstrated to be refractory to AAV treatment (55), intravenous administration of the high dose appeared to at least partly mitigate this problem. Here, we found a reduction in oligodendrocyte numbers that contrasted with findings in GM1 cats (54). This could be due to the earlier disease onset of the SD versus the GM1 cat model, and there is an emerging consensus that oligodendrocyte pathology occurs early in the disease cascade (55, 56). We found oligodendrocyte and myelin pathology to be less refractory to AAV treatment than in SD mice (56), likely because of differences in species, route of administration (intravenous versus intracranial), and capsid (AAV9 versus AAV1). Because the brainstem is the latest-myelinating brain region during development, the increase in oligodendrocyte numbers in the brainstems above normal levels seen in the long-term cohort, similar to AAV-treated GM1 cats (54), supports the hypothesis that self-correction in the form of oligodendrocyte proliferation is possible if treatment occurs before the pathological cascade has advanced too far (54, 55).

“The earlier, the better” has long been considered an apt axiom for treating many storage disorders, including gangliosidosis. Not only is early treatment expected to address pathology before it fully develops, but gene therapies injected before maturation of the BBB should also penetrate the brain parenchyma more effectively. Most patients are diagnosed only after the onset of clinical signs, that is, at an older age when the BBB is intact. However, a subset of patients is identified presymptomatically, usually after an older sibling is diagnosed. Also, as effective treatments achieve regulatory approval for clinical application, the goal is to include TSD and SD in newborn screening panels to facilitate therapy as soon as possible. Future preclinical studies should test therapies at an age when the BBB has not matured.

Limitations of the project included small group sizes relative to studies in rodent models. Our previous studies found that groups of

three or four cats were sufficient to detect statistical differences in the most important outcome measures, including survival, neurological function, enzyme activity, and storage levels after treatment. Definite conclusions based on these outcome measures were produced in this study, but results were less certain with subtler or more variable readouts (such as inflammatory markers). Another limitation was the lack of reliable behavioral tests for cats, which would enable a more thorough evaluation of cognitive function after AAV treatment. Patients and loved ones justifiably are concerned about long-term cognitive function after treatment. Beyond general observations that treated SD cats behaved similarly to normal controls, we could not demonstrate that cognitive function was preserved by AAV treatment. Also, although intravenous injection of AAV9-Bic-fHex improved the clinical statuses and life spans of SD cats, an alternative catabolic pathway for GM2 degradation identified in mice is thought to function, to a lesser degree, in cats (57). Low-level, HexB-mediated cleavage of GM2 ganglioside is sufficient to prevent most symptoms and permit a normal life span in TSD mice (α subunit deficient) (57–59). Even minimal function of the alternative degradative pathway in cats may exaggerate treatment efficacy expected in humans, which use only the classic, HexA-mediated pathway for GM2 catabolism.

This study demonstrates safety and dose-dependent efficacy of AAV9-Bic-fHex in the feline model of SD, and it supports translation of the vector into early-phase clinical trials. Because both subunits of Hex are expressed, both TSD and SD can be treated with a single vector. Deficiency of either subunit destroys the function of HexA ($\alpha\beta$), the only isozyme that degrades GM2 ganglioside in humans, and this study demonstrates effective HexA production and clinical efficacy from the bicistronic vector in a feline model. In addition, the variability in longevity, clinical signs, and Hex activity in the long-term groups (of both doses) supports the hypothesis that AAV treatment shifted the phenotype in SD cats from one of early onset to that of a delayed-onset form. Because disease severity correlates inversely with the degree of residual HexA activity, introducing functional copies of *HEXA* and *HEXB* cDNAs into SD cats and modestly increasing Hex activity created a crude representation of a delayed-onset phenotype. The longer treated cats lived, the more heterogeneous their life spans and clinical signs. Therefore, AAV-treated SD cats may provide an opportunity to better understand delayed-onset phenotypes of GM2 gangliosidosis, an important step to optimal treatment strategies. Although intravenous delivery of AAV9-Bic-fHex was efficacious, extending the life spans of SD cats almost threefold, gene therapy must continue to evolve for maximal benefit in patients. Subsequent administration(s) of vector is one solution, but the development of anti-capsid titers in treated cats means that readministration of vector will be ineffective or even dangerous. Therefore, improvements in AAV treatment of TSD and SD are likely to come from optimized vector design and early intervention.

First reported in 1881, GM2 gangliosidosis remains untreatable today (60). Our study provides an important step toward the goal of minimally invasive, meaningful treatment of these devastating conditions. Clinical trials will provide the ultimate assessment of vector safety and efficacy in patients.

MATERIALS AND METHODS

Study design

The objective of the study was to test intravenous administration of a bicistronic construct expressing Hex α and β subunits for treatment

of both TSD and SD using a single AAV9 vector. The primary goal was to test efficacy in an established feline model of SD as an indicator of vector suitability for human clinical trials. Animals were treated with low (5×10^{13} vg/kg) and high (2×10^{14} vg/kg) doses, and they were followed short term (16 weeks posttreatment) or long term (to humane end point, the inability to stand). Observation and measurement techniques ranged from clinical (survival, neurological exams, body weight, elastography, MRI, MRS, and body fluid biomarkers) to cellular and molecular [histopathology, enzymatic activity, GM2 ganglioside quantitation, immunohistochemistry (IHC), and reverse transcription quantitative polymerase chain reaction (RT-qPCR) for neuronal and glial markers]. The pathologist who analyzed tissues was blinded to treatment group. Those who collected in-life data were not blinded to treatment status (AAV-treated or untreated control) due to insufficient personnel capable of treatment and downstream follow-up. Untreated SD cats were randomly assigned to the low- or high-dose treatment groups, with at least one male and female in each group. The first cats treated for each dose were assigned to the long-term treatment group to allow the study to conclude in a timely manner. A sample size of four cats per treatment group was predetermined, but limitations in vector manufacturing resulted in one treatment group with $n = 3$ (high dose, short term). Sample size was determined on the basis of the investigators' experience and a power analysis using pilot data for enzyme activity in the cerebellums of SD cats treated with the low dose of AAV9-Bic-fHex compared with that of untreated SD cats ($\alpha = 0.05$, power = 0.95, calculated sample size = 4). Data collection was not stopped because no animals reached predetermined criteria approved by the Auburn University Institutional Animal Care and Use Committee. The ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines were followed. No animals were excluded from data collection or analysis, and no samples were treated as outliers.

AAV vector preparation

Preparation of the AAV9 bicistronic vector was performed similarly to previously published methods of AAV9-Bic in mice (20), except that feline *HEXA* and *HEXB* cDNAs were used in this study. The vector includes a centrally located, bidirectional enhancer/promoter complex consisting of a CMV enhancer flanked by two chicken β -actin promoters in opposite directions (fig. S9). The low and high doses were 5×10^{13} vg/kg and 2×10^{14} vg/kg, respectively.

Animals and AAV treatment

A breeding colony of SD cats is maintained at the Scott-Ritchey Research Center at Auburn University, and all experiments in this study were approved by its Institutional Animal Care and Use Committee. Affected SD cats were treated between 0.77 and 1.47 months of age (mean, 1.10 ± 0.15) and 0.38 and 0.70 kg body weight (mean, 0.49 ± 0.15). An intravenous catheter was placed in the cephalic vein and flushed with saline. Vector was delivered slowly over a period of 1 to 2 min via the catheter, which was subsequently flushed with saline. Cats were monitored for at least 3 hours posttreatment for any adverse reactions. Immune suppression was not attempted due to a lack of effective, standardized protocols in cats and the appearance of side effects in previous attempts to immunosuppress cats. Euthanasia was performed with cardiac injection of pentobarbital, and animals were subsequently perfused with heparinized saline. AAV-treated cats were euthanized 16 weeks posttreatment (low dose, $n = 4$; and high dose, $n = 3$) or followed until they reached the

humane end point of inability to stand (low dose, $n = 4$; and high dose, $n = 4$). Controls were age-matched untreated normal cats ($n \geq 3$) and untreated SD cats at the same humane end point ($n \geq 3$).

A 10-point scale based on gait dysfunction in untreated SD cats (9) was used to track disease progression. Age of symptom onset $\pm$ standard deviation are shown in parentheses: 10, normal ($<1.3 \pm 0.2$ months); 9, hindlimb weakness (2.1 ± 0.0 months); 8, wide stance (2.2 ± 0.4 months); 7, ataxia (2.5 ± 0.3 months); 6, instability with occasional falling (2.9 ± 0.5 months); 5, limited walking (3.5 ± 0.5 months); 4, can stand but not walk (3.9 ± 0.5 months); and 3, cannot stand (humane end point, 4.3 ± 0.2 months for untreated SD cats). One point was subtracted for acquisition of each of the following: fine tremors, full-body tremors, hindlimb spasticity, and forelimb spasticity.

Tissue preparation

Brains were divided into nine transverse blocks of 0.6 cm from the frontal pole through the cerebellum (Fig. 5). Blocks from the right hemisphere were frozen in optimal cutting temperature medium and used for measuring specific enzyme activity by synthetic fluorogenic substrates based on 4-methylumbelliferone as well as qPCR for AAV vector biodistribution. Blocks from the left hemisphere were halved to 0.3 cm and either flash frozen in liquid nitrogen or fixed in 10% formalin. Frozen tissue was stored at -80°C and used for high-performance thin-layer chromatography (HPTLC) assays to quantify storage material. Formalin-fixed tissue was embedded in paraffin blocks and used for Luxol Fast Blue or IHC staining.

Ultrasound elastography

Tissue elasticity was assessed with two-dimensional shear wave elastography with acoustic radiation impulse force as previously described in the feline liver (48, 61), kidney (47, 61), and spleen (61). The semitendinosus was also assessed as a representative of skeletal muscle. Cats were sedated as needed while a Toshiba Aplio 500 ultrasound machine with a 15L5 linear probe was applied to the organ of interest. Shear wave speed was quantified as Young's modulus to determine tissue elasticity value (kilopascals). For the liver, measurements were recorded from six to eight intralobular sites from the right, central, and left portions, which were then averaged together. Large vascular and ductal structures were avoided. Care was taken so that shear wave sampling region of interest and depth were consistent between cats.

MRI and MRS

Cats were placed under general anesthesia with dexmedetomidine and ketamine and maintained with isoflurane. MRI and MRS data were collected as previously described (54, 62) on a 7-T scanner (Siemens Healthcare) on SD cats immediately before necropsy ($n = 3$ for untreated and $n = 15$ for all treated cats) and on normal cats at 4 to 5, 8, and 12 months of age ($n = 3$ per time point). To acquire MRS data, voxels were placed in the thalamus (7 mm by 6 mm by 8 mm), parietal cortex (7 mm by 6 mm by 8 mm), and cerebellum (7 mm by 7 mm by 8 mm). MRS data were processed with LC model and internal water scaling (www.s-provencher.com/lcmodel.shtml).

Statistical analysis

All statistical analyses and graphs were generated with GraphPad Prism version 9.1.2 for Mac OSX (GraphPad Software). With the exception of survival analysis, where Mantel-Cox test was used, all tests were

ordinary one-way analysis of variance (ANOVA) with Fisher's least significant difference uncorrected post hoc tests, with the only comparisons made between (i) untreated SD and all other groups or (ii) low dose versus high dose. A data file for all figures with experimental groups of $n < 20$ is included in the Supplementary Materials (data file S1).

Supplementary Materials

The PDF file includes:

Materials and Methods
Figs. S1 to S9
Legend for data file S1
Reference (63)

Other Supplementary Material for this manuscript includes the following:

Data file S1
MDAR Reproducibility Checklist

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Intravenous gene therapy improves life span and clinical outcomes in a feline model of Sandhoff disease

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