Molecular Therapy
Original Article

Longitudinal *In Vivo* Monitoring of the CNS Demonstrates the Efficacy of Gene Therapy in a Sheep Model of CLN5 Batten Disease

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Neuronal ceroid lipofuscinoses (NCLs; Batten disease) are neurodegenerative lysosomal storage diseases predominantly affecting children. Single administration of brain-directed lentiviral or recombinant single-stranded adeno-associated virus 9 (ssAAV9) vectors expressing ovine *CLN5* into six pre-clinically affected sheep with a naturally occurring CLN5 NCL resulted in long-term disease attenuation. Treatment efficacy was demonstrated by non-invasive longitudinal *in vivo* monitoring developed to align with assessments used in human medicine. The treated sheep retained neurological and cognitive function, and one ssAAV9-treated animal has been retained and is now 57 months old, almost triple the lifespan of untreated CLN5-affected sheep. The onset of visual deficits was much delayed. Computed tomography and MRI showed that brain structures and volumes remained stable. Because gene therapy in humans is more likely to begin after clinical diagnosis, self-complementary AAV9-*CLN5* was injected into the brain ventricles of four 7-month-old affected sheep already showing early clinical signs in a second trial. This also halted disease progression beyond their natural lifespan. These findings demonstrate the efficacy of *CLN5* gene therapy, using three different vector platforms, in a large animal model and, thus, the prognosis for human translation.

INTRODUCTION

Batten disease (neuronal ceroid lipofuscinoses [NCLs]) are fatal inherited lysosomal storage diseases affecting the CNS and are caused by mutations in any of 13 different genes, designated *CLN1–CLN8* and *CLN10–CLN14* (http://www.ucl.ac.uk/ncl). Despite their genetic diversity, NCLs are defined by similar pathological and clinical features that include progressive neuronal loss, retinal degeneration, seizures, and psychomotor decline, culminating in premature death.[1] Apart from a recently approved enzyme replacement therapy for the CLN2 disease (cerliponase alfa, BioMarin Pharmaceutical), there are no effective treatments, although several gene and enzyme replacement therapy trials are underway (NCT01161576, NCT01414985, NCT02485899, and NCT02725580; https://clinicaltrials.gov) following studies in animal models.[2–6]

Disease-relevant animal models are invaluable for the development and validation of therapies. However, encouraging preliminary data from gene therapy studies in murine forms[2,3,7,8] need careful consideration before proceeding to translation in human medicine because there are serious caveats with studies in mice. The lissencephalic mouse brain lacks major neuroanatomical structures prominent in the human brain and is very much smaller, 0.4 g versus 1.4 kg, and the neuropathology in many NCL mouse models varies from that seen in the analogous human diseases.[9] Studies using larger animal models with a CNS, physiology, and genomic organization comparable with humans should prove more informative.[10]

Sheep with naturally occurring disease provide ideal models, being similarly sized as humans, weighing 3.5–4.5 kg at birth and growing to 80- to 110-kg adults. The gyrencephalic ovine brain is similar in physical organization to the human brain and in size to non-human primate brains, providing a good approximation for dose requirements, vector distribution, and the utility of gene therapy strategies in pediatric neurodegenerative disorders. The executive function and cognitive capabilities of sheep are now being recognized,[11–13] and their longevity (9–12 years) allows monitoring of the clinical effectiveness and the longer-term consequences of gene therapies over a time frame relevant to disease. Because such trials take several years, and the downstream consequences of successful treatments will require monitoring, there is a pressing need to harvest translational data *in vivo* and allow successful trials to continue without prematurely sacrificing animals for *post mortem* neuropathological evaluation.

Received 21 January 2018; accepted 12 July 2018;
https://doi.org/10.1016/j.ymthe.2018.07.015.

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This study aimed to characterize disease progression in Borderdale sheep with naturally occurring CLN5 and to monitor the efficacy of *CLN5* gene therapy longitudinally through a suite of *in vivo* tests. Affected sheep share the main neuropathological features of the human disease, including progressive brain atrophy and loss of vision from 11–12 months of age, and typically decline to a humane endpoint before 22 months.[14,15] The *in vivo* data presented here show that both single-stranded adeno-associated viral serotype 9 (ssAAV9) and lentivirus-mediated (LV) ovine *CLN5* gene transfer to pre-clinically affected CLN5 lambs prevented stereotypical disease development for an extended period, the only clinical sign observed being delayed-onset visual loss. Furthermore, delivery of self-complementary (sc) AAV9-*CLN5* to affected sheep at an early stage of clinical disease (7 months) attenuated disease progression and brain atrophy to beyond the lifetime of untreated affected sheep. Widespread transduction of the brain and spinal cord was observed in all treated sheep, regardless of the vector platform, *post mortem*. These data provide a strong rationale for clinical translation to CLN5-affected human patients.

RESULTS

Tolerance of *CLN5* Gene Transfer by Pre-clinically Affected Sheep

Six $CLN5^{-/-}$ affected sheep received both intracerebroventricular (i.c.v.) and intraparenchymal (IP) injections of ssAAV9 (n = 3) or LV (n = 3) vectors expressing ovine *CLN5* at 2–3 months of age, before the onset of clinical disease (Table 1). Their rectal temperatures, pulse, and respiratory rates remained normal throughout the 3-week post-injection observation period, and no behavioral changes or clinical signs of an immune response were observed in these sheep, monitored daily for 2 years after these single neurosurgical procedures. All six treated sheep remained free of disease signs when aged 26–27 months, except for a much delayed onset of visual deficits, and well exceeded the ~22-month maximal life expectancy of untreated $CLN5^{-/-}$ sheep. Five were euthanized for neuropathological assessment at this age, and one ssAAV9-treated sheep remains alive in the field, albeit blind, at 57 months of age.

Clinical Efficacy of *CLN5* Gene Transfer to Pre-clinically Affected Sheep

The long-term clinical benefit was assessed monthly against CLN5 heterozygous ($CLN5^{+/-}$) and untreated $CLN5^{-/-}$ sheep using an ovine-specific Batten disease rating scale (oBDRS; Table S1), similar to those used for human NCLs.[16,17] As expected for a recessive disease, $CLN5^{+/-}$ carriers were clinically normal throughout the study, consistently scoring 40 (Figure 1A). Sheep treated before the development of any disease signs also showed no functional deficits, being clinically indistinguishable from healthy $CLN5^{+/-}$ controls for the first 18 months, whereas the oBDRS scores of the untreated $CLN5^{-/-}$ sheep decreased to under 20, reflecting the natural progressive disease course.

This successful trial was extended to determine any longer-term consequences following treatment. A much-delayed loss of vision, apparent by 27 months, was the only disease manifestation noted. Funduscopic examination revealed intermediary indicators of retinal damage, mild tapetal hyperreflectivity, and blood vessel attenuation at this time. Concomitant menace response deficits account for the deviation from normal observed on the oBDRS from 19 months (LV) and 21 months (ssAAV9), respectively (Figures 1A and 1B). Much delayed functional blindness (absent menace response) ensued (untreated $CLN5^{-/-}$ sheep, 12.6 ± 0.4 months; LV-treated sheep, 20.7 ± 0.7 months; ssAAV9-treated sheep, 24.8 ± 1.3 months; $p < 0.0001$) (Figure 1B). At 27 months, pupillary light reflexes were sluggish in the treated sheep, and electroretinography (ERG) responses from the ssAAV9-treated sheep were severely diminished, similar to those seen in untreated $CLN5^{-/-}$ sheep at 15–17 months, and abolished in the LV-treated sheep (Figure S1A).

The treated sheep were otherwise phenotypically normal at 27 months. They were alert and highly interactive with their environment and had developed strong social attachments within their cohort and with animal care personnel, interacting through vocalization and physical contact and detecting the presence of foreign sheep or humans immediately. When grazing in the open field, they showed no evidence of the stereotypical behavior, reduced mentation, wide stance, manifest ataxia, and hindlimb paresis or localized seizure activity seen in the advanced stages of ovine CLN5 disease (Figures 1D–1G).

In stark contrast, stereotypical disease progression had already commenced in the untreated $CLN5^{-/-}$ cohort at 6 months (Figure 1). Initial manifestations were apprehensive walking with low head carriage and balking when in shadows, passing through gateways, or negotiating steps. Vision deteriorated, and by 13 months, untreated animals were functionally blind, exhibiting bilateral absence in menace response, loss of visual tracking, and decreased acoustic startle that affected their ability to flock. Proprioceptive and motor deficits progressed to a wide stance, hindlimb ataxia, stumbling, and intermittent episodes of localized muscle tremors, particularly of the ears, eyelids, lips, and limbs. Behavioral changes included self-segregation, repetitive actions such as aimless circling, and feeding abnormalities, including dribbling and sham grazing. From 15 months, they lost body condition and demonstrated low mentation, extended periods of somnolence, and poor or no responses to a variety of stimuli, including loud noises, flashing lights, and approaching humans. Extremely hyperreflective foci in the retina were obvious to the naked eye, and fundoscopy showed thinned retinal vasculature, together indicative of photoreceptor damage. The neurological phenotype from 17 months was accompanied by tremors ranging from subtle to whole-body inducible seizures, defining their humane endpoint.

One of the ssAAV9-*CLN5*-treated sheep is still alive in the field at 57 months. Her menace response was diminished at 26 and absent at 27 months, ERG amplitudes were extinguished by 29 months, and she developed a pronounced head tilt. She was otherwise clinically normal until 38 months, and then she began to show increased somnolence and sitting and episodes of reduced awareness and

Table 1. Treatment Groups and Current Clinical Status of Treated Sheep

	Sheep	Genotype	Treatment[a]	Treatment Age (Months)	Total Dose[b]	Delivery Route[c]	Gender[d]	Endpoint or Current Age (Months)[e]	Clinical Description	Seizures
Trial 1 controls	1,103	*CLN5*$^{+/-}$	untreated	NA	NA	NA	M	18.4	normal	–
	1,118	*CLN5*$^{+/-}$	untreated	NA	NA	NA	M	19	normal	–
	1,121	*CLN5*$^{+/-}$	untreated	NA	NA	NA	M	18.7	normal	–
	1,128	*CLN5*$^{+/-}$	untreated	NA	NA	NA	M	57.8	normal	–
	1,107	*CLN5*$^{-/-}$	untreated	NA	NA	NA	M	17.2	deceased, blind, advanced disease	+
	1,114	*CLN5*$^{-/-}$	untreated	NA	NA	NA	M	18.3	deceased, blind, neurological disease	–
	1,115	*CLN5*$^{-/-}$	untreated	NA	NA	NA	M	18.8	deceased, blind, advanced disease	+
	1,116	*CLN5*$^{-/-}$	untreated	NA	NA	NA	M	17.3	deceased, blind, advanced disease	+
Trial 1	1,106	*CLN5*$^{-/-}$	LV-MND-*CLN5*	2.1	3.6×10^9	i.c.v./IP	W	26.9	deceased, blind	–
	1,117	*CLN5*$^{-/-}$	LV-MND-*CLN5*	2.2	3.6×10^9	i.c.v./IP	W	27.0	deceased, blind	–
	1,126	*CLN5*$^{-/-}$	LV-MND-*CLN5*	2.4	3.6×10^9	i.c.v./IP	F	27.1	deceased, blind	–
	1,105	*CLN5*$^{-/-}$	ssAAV9.MND.*CLN5*	3.5	3.1×10^{12}	i.c.v./IP	F	27.1	deceased, onset of visual deficits	–
	1,109	*CLN5*$^{-/-}$	ssAAV9.MND.*CLN5*	3.4	3.1×10^{12}	i.c.v./IP	F	57.0	alive, blind	–
	1,120	*CLN5*$^{-/-}$	ssAAV9.MND.*CLN5*	2.8	3.1×10^{12}	i.c.v./IP	F	26.4	deceased, blind	–
Trial 2 controls	1,100	*CLN5*$^{+/-}$	untreated	NA	NA	NA	F	23.8	normal	–
	1,105	*CLN5*$^{+/-}$	untreated	NA	NA	NA	F	23.7	normal	–
	1,106	*CLN5*$^{+/-}$	untreated	NA	NA	NA	F	23.7	normal	–
	1,109	*CLN5*$^{-/-}$	untreated	NA	NA	NA	F	23.2	deceased, blind, advanced disease	+
	1,110	*CLN5*$^{-/-}$	untreated	NA	NA	NA	F	18.3	deceased, blind, advanced disease	+
	1,122	*CLN5*$^{-/-}$	untreated	NA	NA	NA	F	18.1	deceased, blind, advanced disease	+
Trial 2	1,164	*CLN5*$^{-/-}$	scAAV9.CBh.*CLN5*	7.5	4.0×10^{12}	i.c.v.	F	32.3	alive, blind	–
	1,165	*CLN5*$^{-/-}$	scAAV9.CBh.*CLN5*	7.5	4.0×10^{12}	i.c.v.	F	22.3	deceased, blind	–/+
	1,170	*CLN5*$^{-/-}$	scAAV9.CBh.*CLN5*	7.3	4.0×10^{12}	i.c.v.	F	32.1	alive, blind	–
	1,172	*CLN5*$^{-/-}$	scAAV9.CBh.*CLN5*	7.3	4.0×10^{12}	i.c.v.	F	22.5	deceased, visual deficits	–

[a]LV, lentivirus vector; AAV9, adeno-associated viral vector serotype 9; ss, single-stranded; sc, self-complementary; MND, myeloproliferative sarcoma virus enhancer, negative control region deleted, dl587rev primer-binding site substituted promoter; CBh, hybrid chicken β-actin promoter.
[b]Transducing units (TUs) for LV; viral genomes (vgs) for AAV9.
[c]i.c.v., intracerebroventricular; IP, intraparenchymal.
[d]M, male; F, female; W, wether (castrated male).
[e]Untreated sheep were euthanized at their clinical humane endpoint. Treated sheep (boxed) remain alive unless indicated.

depression. Mild stereotypical behavior, including aimless wandering, followed from 42 months (Figures 1F and 1G). Her oBDRS clinical rating score plateaued at 43 months and remains at 22 currently, equivalent to that of untreated *CLN5*$^{-/-}$ animals at 16 months (Figure 1). At a healthy 80 kg, she has not shown the condition loss typical of untreated *CLN5*$^{-/-}$ sheep, still exhibits social and exploratory behavior, recognizes familiar human voices, and takes advantage of her weight and sense of smell to push much younger flockmates aside to receive pelleted food rewards. She moves well within the control flock without hitting obstacles, but her lack of vision becomes obvious when she is isolated and she can run directly into fences when stressed.

Functional Efficacy of *CLN5* Gene Transfer to Pre-clinically Affected Sheep

Cognitive decline was assessed in a closed field maze test that relied on the natural flocking instinct of sheep to motivate passage to conspecifics penned in a goal area[18,19] (Figures 2A and 2B). A food incentive was added to encourage traversal because sheep can become habituated to human contact and lose their natural

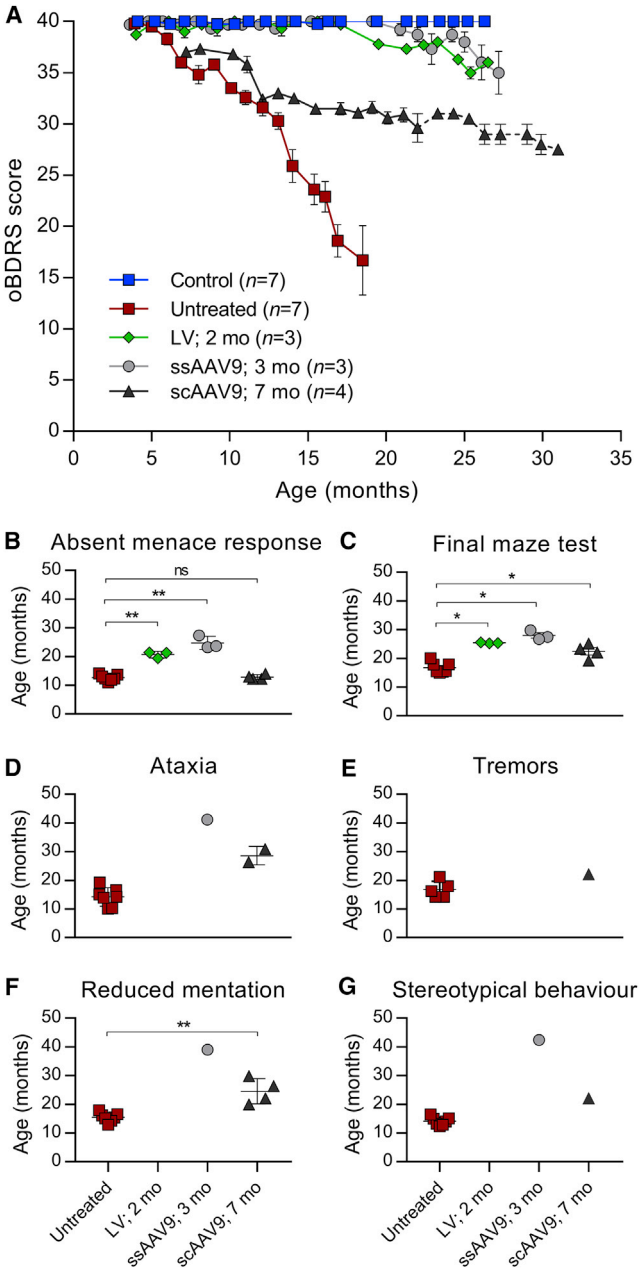

Figure 1. *CLN5* Gene Therapy Provides Long-Term Clinical Efficacy to CLN5-Affected Sheep

(A) *CLN5*$^{-/-}$ sheep that received pre-clinical LV or ssAAV9-*CLN5* gene therapy (2–3 months) or scAAV9-*CLN5* at an early clinical age (7 months) were assessed on the multimodal ovine Batten disease rating scale (oBDRS; Table S1) and compared against healthy *CLN5*$^{+/-}$ and untreated *CLN5*$^{-/-}$ controls over time. Lower scores reflect greater impairment. (B–G) The onset of phenotypic disease traits were assessed, including (B) absent menace response, (C) maze test failure, (D) ataxia, (E) tremors, (F) reduced mentation, and (G) stereotypical behavior in the LV-, ssAAV9-, and scAAV9-treated cohorts and untreated *CLN5*$^{-/-}$ sheep. Each point represents the age of onset for an individual sheep. Missing points indicate that the sheep did not yet exhibit the trait during the study. The dashed portion of the line for the scAAV9-treated sheep arises from data from the two animals still alive. All data are expressed as means ± SEM. *p < 0.05, **p < 0.01, nonparametric Mann-Whitney test where appropriate.

wariness. No difference in maze performance was discernible between treated sheep and healthy *CLN5*$^{+/-}$ sheep for many months after untreated *CLN5*$^{-/-}$ sheep reached end-stage disease (Figure 2A). Treated sheep walked or ran through the maze with their heads held high, avoiding the obstacles and error zones, at traverse times usually faster than the heterozygote controls. The effects of their treatment-delayed visual loss surfaced from 24 and 25 months for the LV and AAV9 cohorts, respectively (Figure 1). Traverse times and failure rates increased (Figures 2A and 1C), and they began to collide with maze obstacles.

Untreated *CLN5*$^{-/-}$ sheep showed progressive navigational decline (Figure 2A) and an affected phenotype from an early age. Despite their deteriorating vision (Figure 1B), they were able to negotiate the maze for another few months (Figure 1C), albeit at progressively longer traverse times. By 16 months, they failed to transit the error zone or became too akinesic to complete the maze, and testing was abandoned. They still tended to avoid obstacles, pulling up short, but failed to find a way around.

Maintenance of Brain Volume and Structure after *CLN5* Gene Transfer to Pre-clinically Affected Sheep

Three-dimensional reconstructions from longitudinal computed tomography (CT) scanning were used to monitor brain atrophy *in vivo*[20] and revealed a corrective effect of pre-clinical *CLN5* gene therapy (Figure 3). Treated brains maintained the structural appearance of healthy control brains, with no evidence of the atrophy characteristic of untreated *CLN5*$^{-/-}$ brains, particularly the notable degeneration of the occipital and parietal cortices that generalizes to the entire cortical mantle by 19 months.

Treatment with either ssAAV9 or LV vector largely attenuated volume loss, but differences in initial volumes and subsequent rates of change varied between individuals, somewhat obscuring the data (Figure 4A). Differences became much clearer when mean cumulative intracranial volume differences in each group were plotted (Figure 4B). The cranial volumes of the ssAAV9-treated sheep closely followed those of the *CLN5*$^{+/-}$ control animals, and volumetric increases for the LV-treated sheep were even greater. This contrasted with the shrinkage of the untreated *CLN5*$^{-/-}$ brains.

The veracity of the structures derived from CT scanning was confirmed by MRI of treated sheep at 25 months. Overall brain structure and architecture remained normal (Figure 5). Ventricles of treated animals were not noticeably enlarged, nor were overt widening of the subarachnoid cerebrospinal fluid (CSF) spaces or sulcal prominence observed. There was none of the cranial ossification present in younger untreated *CLN5*$^{-/-}$ controls.

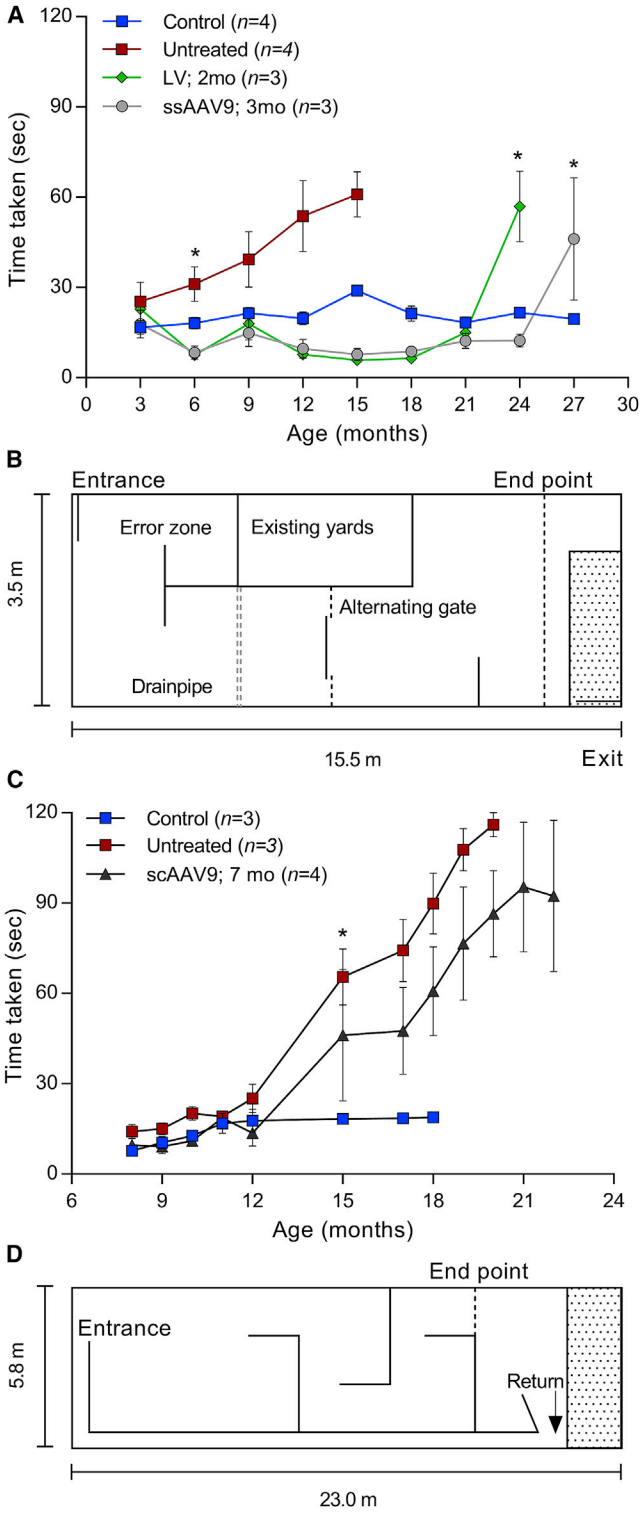

Figure 2. *CLN5* Gene Therapy Delays the Onset of Cognitive Deficits in CLN5-Affected Sheep

Vision and cognition were assessed in closed field mazes. (A) Mean traverse times for sheep treated pre-clinically with LV or ssAAV9-*CLN5* compared with healthy $CLN5^{+/-}$ and untreated $CLN5^{-/-}$ controls (2 runs per animal at each time) in the first maze configuration (B). (C) Mean traverse times for sheep treated with scAAV9-*CLN5* at an early clinical disease stage (5 runs per animal) compared with healthy $CLN5^{+/-}$ and untreated $CLN5^{-/-}$ controls in the second maze configuration (D). All data are the means ± SEM. The earliest times at which traverse times differed between cohorts are indicated. *$p < 0.05$, Student's t test.

Longitudinal CT of the remaining ssAAV9-treated sheep revealed that, after plateauing at 102.4 ± 0.7 mL from 11–27 months, her intracranial volume began to decline. It was 84.2 mL at 51 months, similar to that of a 16-month-old untreated $CLN5^{-/-}$ animal, but the only observable phenotypic traits are mild.

Disease Progression Halted after *CLN5* Gene Transfer to Clinically Affected Sheep

A second trial tested the efficacy of *CLN5* gene therapy in sheep with established disease (Table 1). Four 7-month-old $CLN5^{-/-}$ sheep, with evident brain atrophy and early clinical signs, received i.c.v. injections of an scAAV9 vector expressing ovine *CLN5*. The treatment was well tolerated, and aside from visual loss, there was a paucity of evidence of further disease progression. Two of the four sheep were euthanized at 23 months for neuropathological analyses, whereas the other two remain alive and well at 32 months

Clinical efficacy was monitored monthly on the oBDRS (Figure 1A). These sheep already had a mild clinical phenotype at enrollment, including a slight head tilt and/or low head carriage and wariness of shadows, and they crouched when traveling through a confined gateway. Unlike the sheep treated earlier, vision loss was not delayed. These sheep lacked menace responses by 12–14 months, as do untreated $CLN5^{-/-}$ sheep (Figure 1B). ERG amplitudes were extinguished in one sheep by 14 months, another two retained a response until 20 months, whereas the fourth had positive photopic and scotopic responses to 22 months (Figure S1B). Strikingly, there has been no dramatic advance in motor, neurological, or behavioral dysfunction in this scAAV9-treated cohort from 12 to 32 months. One sheep had short periods of heightened somnolence and exhibited inducible facial tics and mild circling behavior on stress prior to euthanasia at 23 months, but the collective oBDRS scores of the two remaining treated sheep have plateaued just below 30 (Figure 1). To compare, age-matched untreated $CLN5^{-/-}$ flockmates developed stereotypical CLN5 disease with manifest seizures and negative ERGs from 16–21 months, prompting euthanasia at 18–23 months when oBDRS scores ranged from 17.5–11.5 (Figure 1A).

Following relaxation by the New Zealand Environmental Protection Agency regarding the status of AAV9-treated animals, sheep on this second trial were maintained outdoors, enabling more complex maze testing (Figure 2D). The maze traverse times of the untreated $CLN5^{-/-}$ sheep reflected their progressive slowing and ultimate failure, whereas the scAAV9-treated sheep retained speed and the ability to traverse for longer, albeit to varying extents (Figures 2C and 1C). The sheep with ERG amplitudes extinguished at 14 months struggled to complete the maze thereafter, but the other three still completed

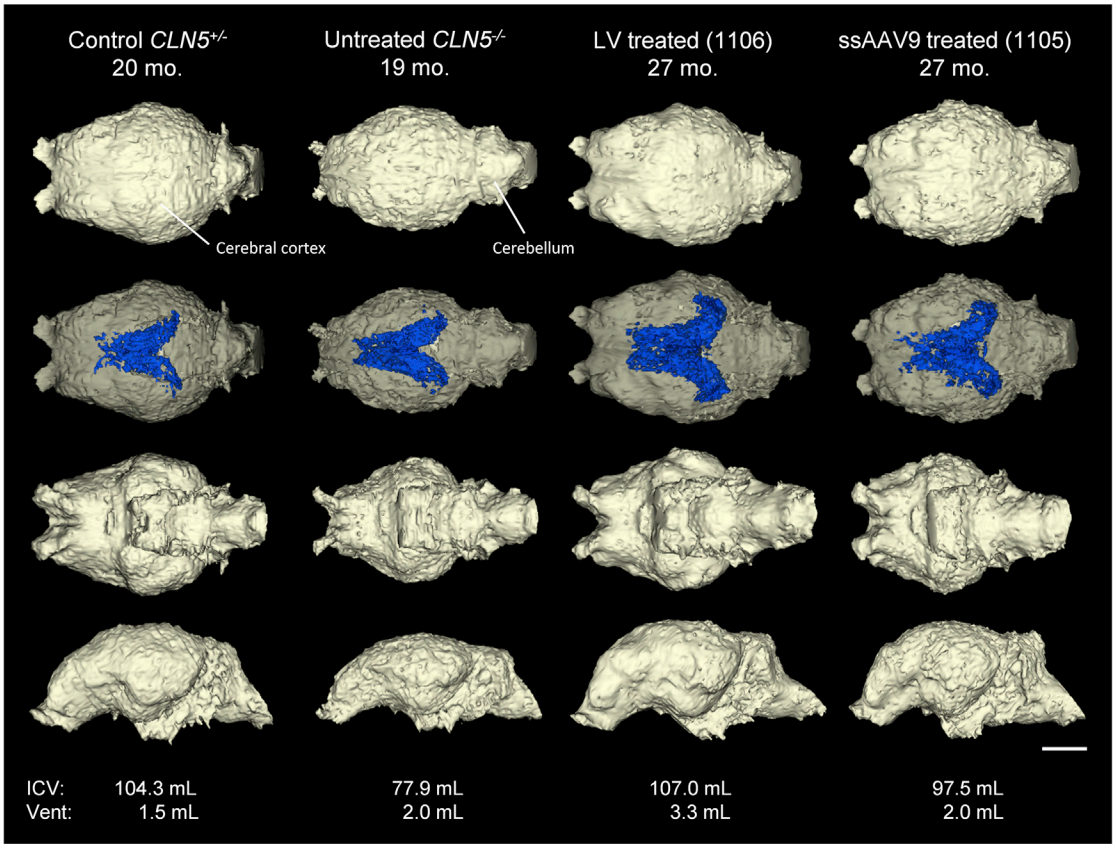

Figure 3. Three-Dimensional Modeling of Gross Neuroanatomy after Pre-Clinical *CLN5* Gene Therapy

Representative three-dimensional models of the sheep cranium and lateral ventricles reconstructed from CT images showing no discernible differences in the gross anatomy of the pre-clinically treated brains and a healthy $CLN5^{+/-}$ control at 27 months. Both lacked the pronounced atrophy visible in the younger untreated $CLN5^{-/-}$ cerebral cortex at 19 months. Note the relative sparing of the affected cerebellum. The scale bar represents 2 cm.

most or all of the five maze runs within the allocated 120 s at 19 months. The two sheep still alive were removed from the monthly testing regime at 24–25 months when they could no longer traverse the maze.

Sheep in this more complex maze wore harness-attached global positioning system (GPS) units to track their movements (Figure 6). Healthy $CLN5^{+/-}$ control sheep followed a uniform track through the maze with few pauses, avoided obstacles, and reached conspecifics in the goal area quickly. Initially, treated sheep also followed the most direct route. With time they found it harder to enter or exit the maze but still completed most of their runs until 24 months. In contrast, untreated $CLN5^{-/-}$ sheep took longer and used a more random path from as early as 8 months. As their disease progressed, they lost any social and/or exploratory behavior and just stood in the maze arena bleating. By 19 months, they circled compulsively in the start area (Figure 6) or were too affected to participate in the testing (Figure 1C).

Longitudinal CT scanning has indicated little post-injection brain atrophy in the scAAV9-treated sheep to date. Intracranial volumes largely plateaued from 89.5 ± 1.2 mL pre-injection to 87.8 ± 0.8 mL at 22 months (all four animals), falling slightly to 85.6 ± 1.7 mL at 32 months (the two remaining animals) (Figures 4A and 4C). Treated brains actually increased in size over the first 3 months post-injection before two periods of mild atrophy and plateaux; hence, cumulative intracranial volume changes have been minimal (−2.5 ± 0.8 mL from injection to 31 months) in contrast to the rapid atrophy seen in untreated $CLN5^{-/-}$ sheep, which causes a loss of 6.7 ± 1.3 mL of intracranial volume from 7 to 19 months. Two scAAV9-treated animals were sacrificed at 23 months to determine the extent of CLN5 expression in the CNS. Immunohistochemistry revealed strong transduction throughout the brain, particularly in the hippocampus and cortical gray matter (Figure 7) as well as in the cerebellum and spinal cord.

DISCUSSION

These trials are the first to demonstrate the efficacy of virus-mediated *CLN5* gene therapy in a large animal model of NCL using outcome measures adapted from human medicine. First, pre-clinically affected sheep were treated with combinatorial IP and i.c.v. injections of either ssAAV used in gene transfer studies into the brain parenchyma in

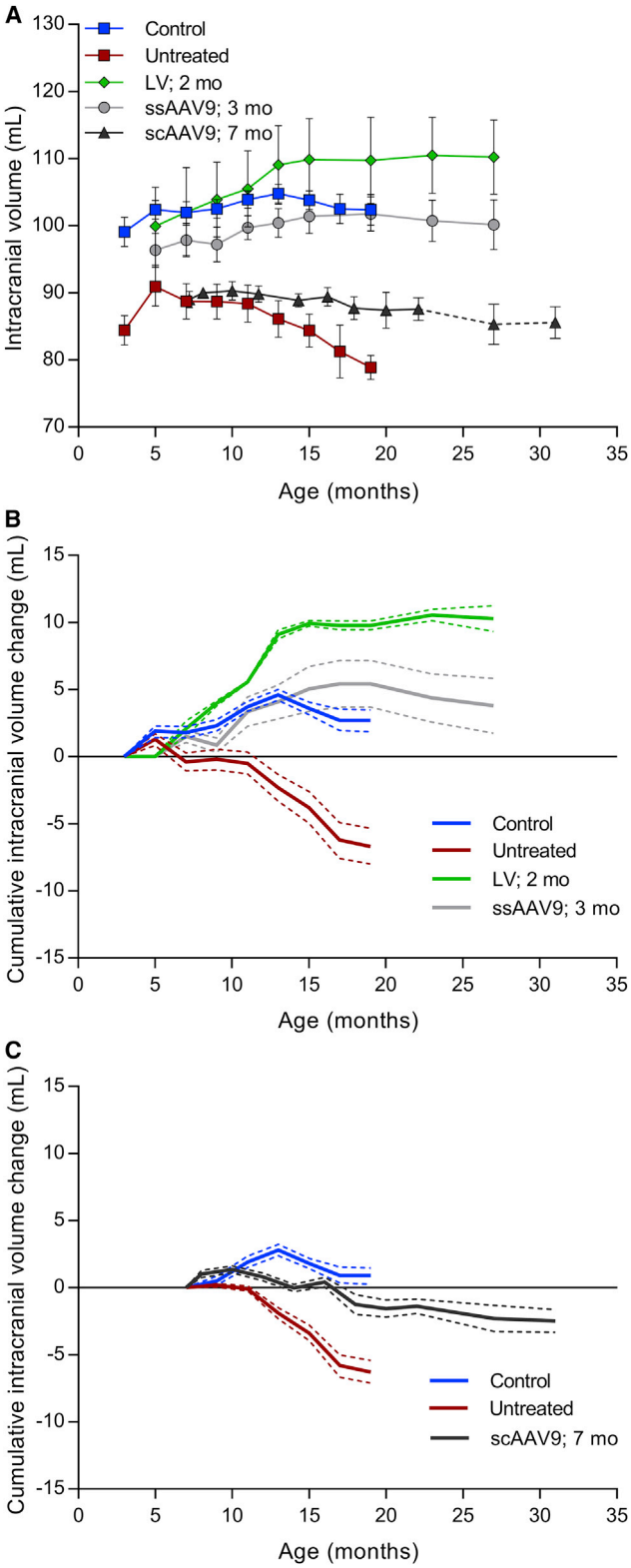

Figure 4. *In Vivo* Neuroimaging Revealed Normalization or Stabilization of Intracranial Volumes after *CLN5* Gene Therapy

Longitudinal CT scans of treated sheep brains compared against control data. (A) Actual scan volumes of sheep brains. (B) Data presented as cumulative volume changes over time post-injection show normalization of intracranial volumes after pre-clinical *CLN5* gene therapy. (C) Data presented as cumulative volume changes over time post-injection show extended volumetric retention in sheep treated at an early clinical stage (7 months) compared with the ongoing atrophy of the untreated affected brains. All data are expressed as means ± SEM.

CLN2 rodent and non-human models[2,3,21,22] or lentiviral vectors that successfully mediated transduction of sheep brains *in* vivo.[23] The ambition was to preferentially treat the CNS using an ubiquitous myeloproliferative sarcoma virus enhancer, negative control region deleted, dl587rev primer-binding site substituted (MND) promoter previously tested in our sheep[23] and doses derived from human, sheep, non-human primate, and rodent studies.[22–24] The original protocol envisaged sacrifice of these animals at 18 months for *post mortem* neuropathological analyses to judge the efficacy of treatment. However, they remained clinically indistinguishable from unaffected controls on the oBDRS at this age (Figure 1), so they were kept alive, and monitoring was continued. Longitudinal neuroimaging and three-dimensional modeling showed that their brain structure, architecture, and size were normalized (Figures 3 and 4). Their quality of life was profoundly improved; they well exceeded the typical lifespan of untreated animals, and there were no signs of disease except late-onset visual deficits that would not have been detected had the trial been terminated at 18 months.

The three LV-treated sheep were euthanized at 27 months following indoor housing restrictions imposed by New Zealand law. AAV9-treated animals were exempted from this restriction at the same time; however, the decision was made to euthanize two of the three ssAAV9-treated sheep at 27 months to allow a comparative *post mortem* assessment of CLN5 expression between the two vectors and to look for pathological correction or any chronic immune response over time. These neuropathological studies are underway. The remaining ssAAV9-treated sheep is still alive and well at 57 months of age, nearly triple the maximal life expectancy of a $CLN5^{-/-}$ animal. Although blind, she lives in the field and does not require the indoor intensive nursing of end-stage $CLN5^{-/-}$ animals. Monitoring for any disease or age-related signs or long-term CNS or peripheral problems continues.

Given that most human cases of NCL only become apparent upon diagnosis following the development of disease symptoms, 7-month-old $CLN5^{-/-}$ sheep with established clinical disease were treated in a second trial. Here the vector platform of choice changed from single-stranded to scAAV vectors following their purported 10- to 100-fold greater transduction efficiency, which potentially reduces the doses required, increases efficacy, and decreases the time between transduction and functional expression.[25–28] The hybrid chicken β-actin promoter (CBh) promoter was chosen for its ability to provide the strong, long-term, and ubiquitous CNS expression shown in other animal studies.[29] The multiple IP injections of the first trial were

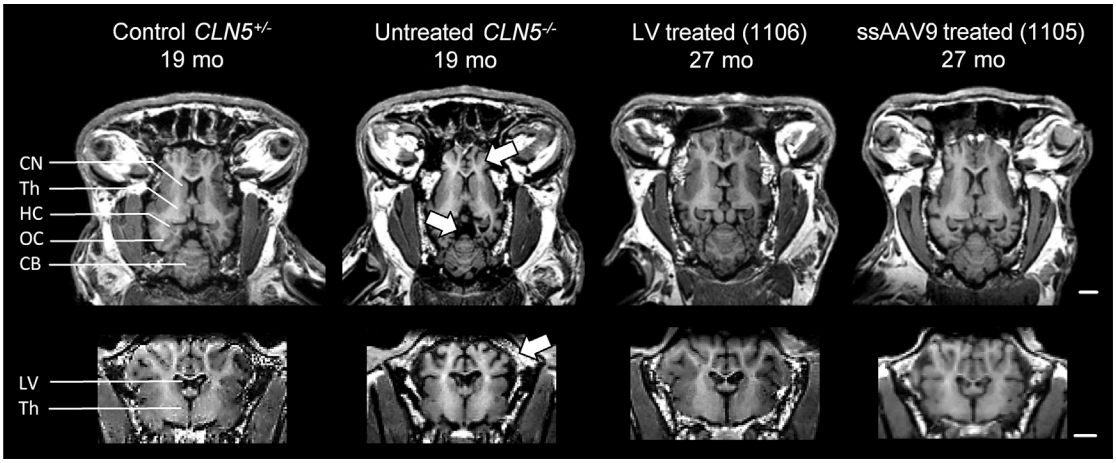

Figure 5. MRI Confirmed Preservation of Neuroanatomical Structure after Pre-clinical *CLN5* Gene Therapy

Volumetric T1-weighted magnetic resonance images in the horizontal (top) and coronal (bottom) views of a 19-month-old healthy *CLN5*$^{+/-}$ control and an untreated *CLN5*$^{-/-}$ sheep brain compared with brains from much older (27 months) sheep that received LV or AAV9-*CLN5* gene therapy before the development of clinical signs. Treatment with either vector preserved neuroanatomical structure, protecting against the profound cortical atrophy (top arrow), prominent ventricular enlargement (center arrow), and cranial thickening (bottom arrow) seen in the untreated *CLN5*$^{-/-}$ brain. The scale bars represent 1 cm. CB, cerebellum; CN, caudate nucleus; HC, hippocampus; LV, lateral ventricle; OC, occipital cortex; Th, thalamus.

abandoned, and sheep in the second trial received i.c.v.-only bilateral injections consequent to the success of this delivery route in other large animal studies.[30,31]

The scAAV9 injections suspended disease progression in these sheep, and two treated sheep remain alive at 32 months. That they went blind at a similar rate as untreated *CLN5*$^{-/-}$ sheep was not unexpected because baseline intracranial volume measurements indicated considerable pre-injection atrophy of the occipital lobe, which continues in untreated *CLN5*$^{-/-}$ sheep (Figure 5).[32] Treatment halted this atrophy, and little further occurred over the following 23 months (Figure 4).

Simple maze tests based on those used in sheep for a number of applications,[33–37] including spatial learning and memory studies,[13] became a valuable adjunct to the clinical studies. Pre-clinically treated sheep retained their ability to navigate the maze well beyond the age at which untreated *CLN5*$^{-/-}$ sheep could not, at traverse times even faster than the unaffected cohort, possibly because they were more habituated to the food reward than the pasture-raised controls. Although they eventually went blind, differences upon clinical examination and end-stage maze behavior (e.g., hitting obstacles) suggest a difference in the pathophysiological cascade initiating visual disturbances from that in untreated *CLN5*$^{-/-}$ animals. Although loss of vision in the untreated *CLN5*$^{-/-}$ sheep paralleled atrophy of the visual cortex noted in histological, MRI,[32] and CT studies (Figures 3, 4, and 5), no such atrophy was observed in the pre-clinically treated brains, even at 27 months. It is likely that the gene therapy prevented the cortical neurodegenerative-driven blindness seen first in untreated *CLN5*$^{-/-}$ sheep but that the vector and/or recombinant CLN5 protein did not penetrate to, or persist in, the retina to prevent its long-term degeneration. Thus, the contemporaneous problems experienced by the treated sheep in navigating the maze were likely to be associated with the later-onset retinal atrophy. This hypothesis is being explored in ongoing neuropathological examinations of the treated sheep brains, which will also reveal any differences in efficacy and CLN5 biodistribution between the various promoters and doses used.

Similar findings were reported in dogs with CLN2 NCL after i.c.v. AAV2-mediated delivery of the soluble lysosomal enzyme tripeptidyl peptidase 1 (TPP1)[30]. Like the sheep, these dogs had an extended lifespan after treatment, with protection from cognitive decline and delayed disease onset and progression and a similar dichotomy of leading symptoms. Treatment did not delay the retinal degeneration in these dogs either.

The performance of certain animal species in cognitive or behavioral tasks, including maze tests, depends on visual ability.[38,39] Here, GPS technology showed that, although post-symptomatically treated sheep went blind and moved slowly, they could still navigate the maze at 22 months of age (Figure 6), suggesting intact spatial memory skills learned when still sighted. Untreated animals lose cognition and, thus, navigational ability well before this age. This technology will be important when testing the efficacy of future combined brain-directed and ocular gene therapies.

Sheep are ideal animals in which to assess the safety, efficacy, and long-term prognosis of gene therapy. They have a large gyrencephalic brain and similar live body weights, spine dimensions, CSF volumes, and pulmonary and cardiac parameters as humans.[40] Presently, sheep models of inherited disease are restricted to flocks arising from cases diagnosed on referral. Completion of the full genome sequence of sheep[41] and the advent of next-generation sequencing technologies

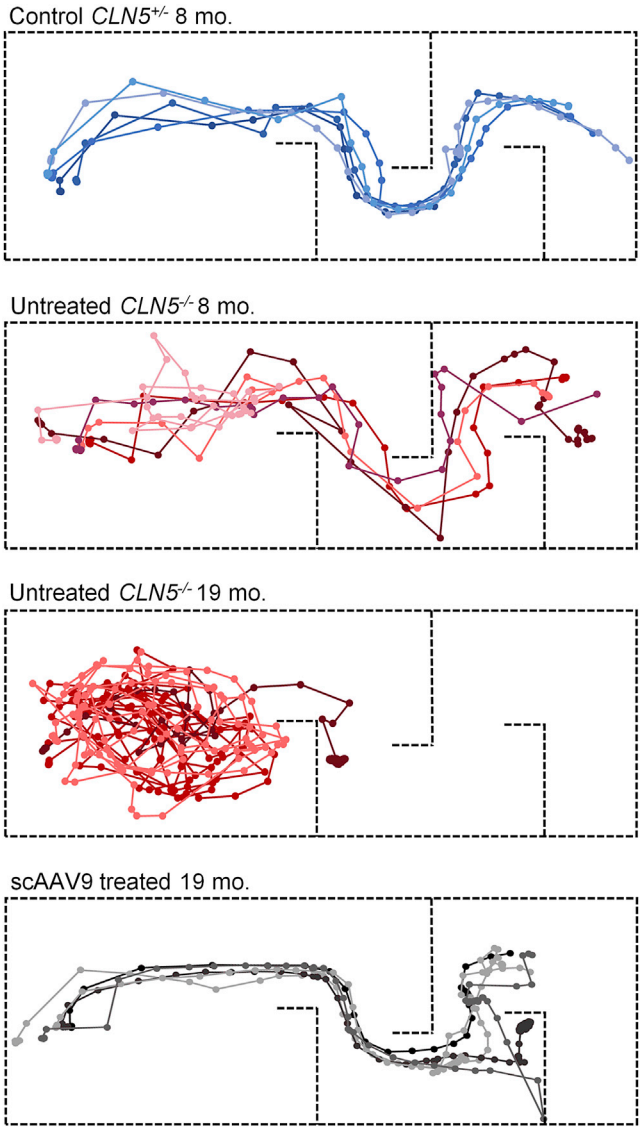

Figure 6. GPS Tracking of Traversal of a Closed Field Maze Highlighted the Delay in Cognitive Decline after *CLN5* Gene Therapy of Clinically Affected Sheep

GPS positional data were collected at 1-s intervals (dots), and five traverses through the close field maze (Figure 2D) were plotted for a representative healthy *CLN5*$^{+/-}$ control, an untreated *CLN5*$^{-/-}$ sheep, and an scAAV9-*CLN5*-treated sheep at different ages. The direct trajectory of the *CLN5*$^{+/-}$ sheep persisted throughout the testing, whereas untreated *CLN5*$^{-/-}$ sheep showed a less uniform path as early as 8 months, with compulsive circling and ultimate failure by 19 months.

make it feasible to screen populations of sheep for genetic variants to yield animal model candidates for an array of diseases.[42] Recent advances in gene modification technologies, such as CRISPR/Cas9 genome editing, are being applied to sheep to generate purpose-built model flocks, increasing the use of this species for model studies. The *in vivo* tests described here will provide robust, quantitative natural history and translational data for these novel sheep models.

Treated sheep may eventually develop disease because of vector attenuation or the protein becoming non-functional with time. These considerations will determine the timing of the eventual *post mortem* neuropathology studies on the treated sheep still alive and give insight into whether medium-term successful treatments could benefit from subsequent re-injections of vector using serotypes chosen to prevent immune rejection. Although intervention at the earliest possible stage will optimize patient outcome, *CLN5* gene therapy to clinically affected animals still had a telling effect, slowing brain atrophy and delaying clinical progression, indicating that clinically affected patients may also benefit.

Our studies so far indicate that ssAAV9, scAAV9, and LV vectors all resulted in effective transduction and amelioration of disease-related pathology. All of the viral cassettes contained the wild-type sheep *CLN5* cDNA sequence with translation from the ovine ATG initiation site in exon 1, which aligns with the third ATG in the human sequence, to yield a 361-amino acid peptide,[15] in line with results of human lysosome proteomic studies.[43] Unfortunately, a number of workers have assumed that the first ATG in the human *CLN5* gene is the start codon, leading to deductions from function and localization studies of uncertain veracity.[44,45] This must be borne in mind when designing vectors expressing the CLN5 protein for human treatments.

In conclusion, these studies establish the efficacy of viral-mediated gene transfer in an ovine CLN5 NCL and provide a particularly pressing case for translation to patients, including those with clinically apparent disease, there being no other treatment options for patients bearing *CLN5* mutations.

MATERIALS AND METHODS

Animals

Borderdale sheep were diagnosed at birth[15] and maintained at Lincoln University under NIH guidelines for the care and use of animals in research and the NZ Animal Welfare Act (1999). All experimental protocols were approved by the Lincoln University Animal Ethics and Institutional Biosafety Committees. Table 1 summarizes the study design. Six *CLN5*$^{-/-}$ pre-clinically affected lambs received bilateral i.c.v. and IP injections of lentiviral (n = 3) or ssAAV9 vectors (n = 3) encoding ovine *CLN5* at 2–3 months and were housed in an indoor Physical Containment 2 sheep facility for 2 years with daily monitoring, in compliance with New Zealand (NZ) Environmental Protection Authority (EPA) requirements. In a second trial, four lambs with clinical signs of disease received bilateral i.c.v. injections of scAAV9-*CLN5* at 7 months and were maintained under normal outdoor pastural conditions following a change in EPA requirements, as were all age-matched *CLN5*$^{+/-}$ unaffected and untreated affected *CLN5*$^{-/-}$ control sheep.

Vectors

LV and recombinant single-stranded AAV9 constructs, encoding ovine *CLN5* (GenBank: NM_001082595) under the control of the myeloid proliferative U3 enhancer element (MND), were produced by the Otago Viral Vector Facility (EPA approval GMDO3091 and GMD101648) for testing in *CLN5*$^{-/-}$ sheep prior to the onset of clinical disease. LVs, pseudotyped with the vesicular stomatitis virus glycoprotein, were

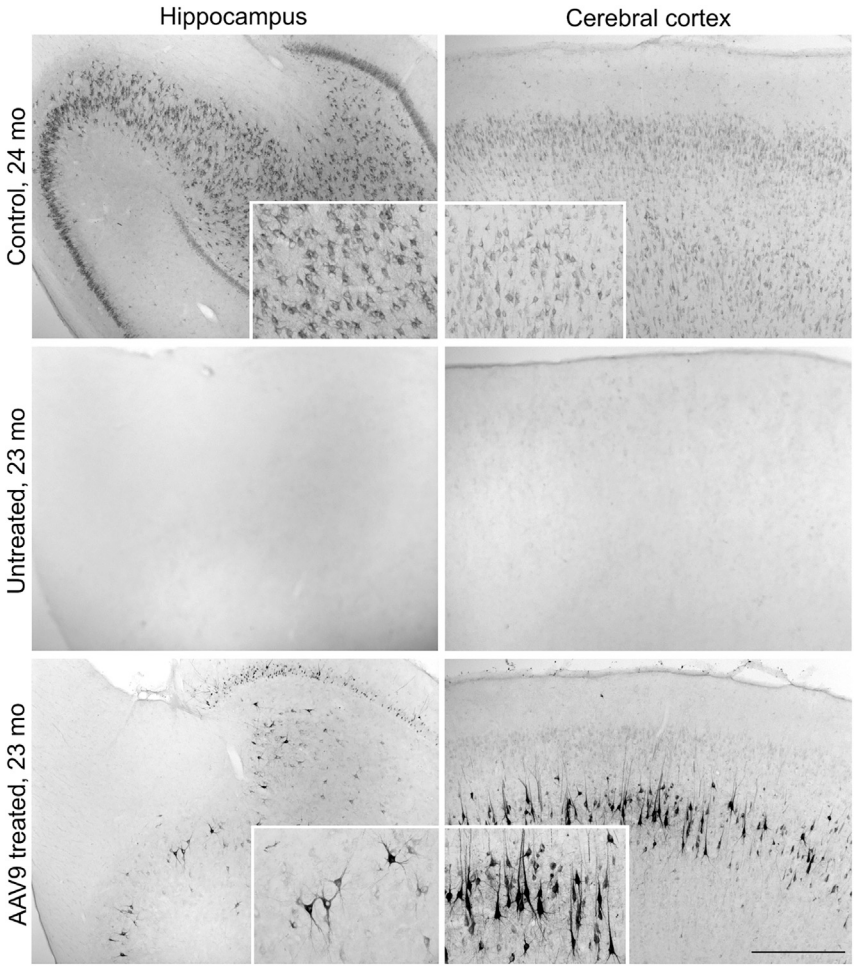

Figure 7. Long-Term Expression of the CLN5 Protein after *CLN5* Gene Therapy

Representative micrographs of the hippocampal and cortical regions in sagittal sheep brain sections of a $CLN5^{+/-}$ control, an untreated $CLN5^{-/-}$ sheep, and a $CLN5^{-/-}$ sheep that received scAAV9-*CLN5* treatment at 7 months, immunostained using an anti-rabbit CLN5 antibody. Endogenous CLN5 protein expression was evident throughout the control brain, which was lacking in the untreated $CLN5^{-/-}$ sheep brain. In contrast, strong CLN5 immunoreactivity was noted in the treated sheep brain over 15 months after intraventricular delivery of scAAV9-*CLN5*. Similar results were seen after pre-clinical delivery of LV or ssAAV9 vectors. CLN5-expressing cells in the treated hippocampal and cortical pyramidal cell layers were morphologically neuron-like. The scale bar represents 500 μm.

packaged using a second-generation packaging system and titrated as described previously.[23] Functional titers were determined by serial dilution on 293T cells, with confirmation by *in vitro* immunofluorescence 72 hr post-transduction. Recombinant ssAAV9-*CLN5* was generated by standard triple transfection methods into 293T cells using packaging and transgene plasmids obtained from the University of Pennsylvania (PA, USA). Recombinant scAAV9, expressing codon-optimized ovine *CLN5* under the control of the CBh promoter (scAAV-*CLN5*), was produced by the University of North Carolina Gene Therapy Center Vector Core (NC, USA) by triple transfection of HEK293 cells, iodixanol gradient centrifugation, and ion exchange chromatography as described previously.[46] Vectors were formulated in 350 mM PBS containing 5% sorbitol, and titers were determined by qPCR.[46]

Vector Delivery

Stereotactic surgical procedures were used as described previously.[23] Pre-clinical $CLN5^{-/-}$ affected sheep received bilateral injections at 2–3 months into the lateral cerebral ventricles and the occipital and parietal cortices via four burr holes. Each lamb received a total dose of either 3.6×10^9 transducing units (TUs) of LV-*CLN5* (110 μL per ventricle; 26 μL per parenchymal site; vector titer, 1.1×10^{10} TUs ml^{-1}) or 3.1×10^{12} viral genomes (vgs) of ssAAV9-*CLN5* (500 μL per ventricle; 25 μL per parenchymal site; vector titer, 2.85×10^{12} vgs ml^{-1}) (Table 1). The four clinically affected $CLN5^{-/-}$ sheep each received bilateral i.c.v. injections of 4.0×10^{12} vgs of scAAV9-*CLN5* (287 μL vector diluted in PBS to a final volume of 400 μL per ventricle; vector titer, 7.0×10^{12} vg ml^{-1}).

Neurological Examination

Physical and neurological clinical assessments were performed monthly on the treated sheep and cohorts of healthy $CLN5^{+/-}$ controls and untreated $CLN5^{-/-}$ sheep. An oBDRS (Table S1) was developed, similar to those used for human NCLs.[16,17] Body condition scores and weights were recorded. Mentation, gait, head carriage, and postural traits as well as manifest tremors or seizure onsets were reported in conjunction with cranial nerve function tests. Each parameter was scored from 4 to 0 (normal to abnormal) by two blinded investigators based on the degree of deviation from healthy function; scores were averaged when there was a discrepancy and then combined to give a total out of 40.

ERG

A single ERG session was performed on the pre-clinically treated sheep when they were 27 months old. scAAV9-treated sheep in the second trial were tested every second month against untreated $CLN5^{+/-}$ and $CLN5^{-/-}$ controls. Mixed receptor response ERG recordings were obtained from each eye after 5 min of dark adaptation using a veterinary ERG system (Eickemeyer, Tuttlingen, Germany).

Maze Testing

Sheep cognition was assessed monthly via performance in a closed field maze (Figure 2B) under daytime photopic light, and the times required to traverse the maze were recorded. Sheep negotiated the

maze twice on each testing day. Testing in the second, more complex maze (Figure 2D) was expanded to five runs per day, and sheep were fitted with harness-attached GPS loggers to collect their position in the maze arena every second. Monthly testing continued until the sheep were unable to complete the maze.

Quantitative Analysis of Brain Atrophy

Brain CT imaging of sheep anesthetized by intravenous injection of 0.8 mg/kg live-weight diazepam (Pamlin injection, Troy Laboratories NZ, Auckland, NZ) and 17 mg/kg live-weight of ketamine hydrochloride (Phoenix ketamine injection, Phoenix Pharm Distributors, Auckland, NZ) was carried out bimonthly. Coronal slices were acquired at 1-mm intervals, 120 kV, 100 mA, 2 s rotation time on a GE Prospeed CT scanner (GE Healthcare, Hyugo, Japan). Three-dimensional modeling and intracranial volumetrics were performed using the three-dimensional slicer 4.3.1 freeware (https://www.slicer.org/).[47] That intracranial volumes are good surrogates for brain volumes was confirmed by comparing the scanned volumes of healthy control (n = 14) and NCL-affected sheep (n = 11) *post mortem* with the volumes of the removed brains, measured by water displacement (K.N.R., N.L.M., N.G. Anderson, C.R. Bunt, M.P.W., T.R.M., G.K.B., D.N.P., unpublished data).

A single structural T1-weighted magnetic resonance image of each sheep brain was acquired on a 3-Tesla GE HDxt MRI scanner (GE Healthcare, Waukesha, WI, USA) with an eight-channel head coil (spoiled gradient recalled echo brain volume imaging (BRAVO) acquisition, echo time/repetition time/interval time (TE/TR/TI) = 5.18/12.44/400 ms, flip angle = 15°, field of view (FOV) = 200 mm, acquisition matrix = 256 × 256, slice thickness = 0.8 mm, voxel size = 0.78 × 0.78 × 0.8 mm^3, scan time = 6:24).

Immunohistochemistry

Methods were similar to those described previously.[23] Brains were perfusion-fixed via the carotid artery with 10% formalin in 0.9% NaCl *post mortem*. After equilibration in 20% sucrose and 10% ethylene glycol in 0.9% NaCl for 7 days, brain hemispheres were frozen at −80°C. Sequential 50-μm sagittal sections were cut on a sliding microtome (Microm International, Walldorf, Germany) and stored at −20°C in 96-well plates in cryoprotectant (PBS) containing 30% ethylene glycol, 15% sucrose, and 0.05% sodium azide).

Antibodies were diluted in 10% normal goat serum (NGS) in PBS (pH 7.4) containing 0.3% Triton X-100 (Sigma-Aldrich) (PBST). Test sections were thawed and blocked with 1% H_2O_2 in PBST for 30 min and then for 1 hr in 15% NGS prior to overnight incubation in rabbit polyclonal anti-CLN5 (Dr. Stephanie Hughes, University of Otago; 1:500). Immunoreactivity was detected using biotinylated goat anti-rabbit immunoglobulin G (IgG) (Sigma-Aldrich, B7389; 1:1,000) for 4 hr, followed by ExtrAvidin peroxidase (Sigma-Aldrich, E2886; 1:1,000) for 4 hr. Staining was visualized using 0.5 mg/mL 3,3′-diaminobenzidine (DAB; Sigma-Aldrich, D5637) and 0.01% H_2O_2 in PBS.

Statistics

One-way ANOVA was used to assess the statistical significance of differences between the control and treatment groups (GraphPad Prism 7, GraphPad, La Jolla, California, USA). Pairwise comparisons were then performed between treatment groups using the Mann-Whitney test or Student's t test as indicated. Data are expressed as means ± SEM, and $p \leq 0.05$ was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and one table and can be found with this article online at https://doi.org/10.1016/j.ymthe.2018.07.015.

AUTHOR CONTRIBUTIONS

N.L.M., S.M.H., S.J.G., and D.N.P. designed the research and vectors. L.S., H.E.W., S.M.H., and S.J.G. prepared the vectors. N.L.M., K.N.R., M.P.W., G.K.B., and T.R.M. performed the research and analyzed the data. N.L.M. and D.N.P. wrote the paper.

CONFLICTS OF INTEREST

No authors have conflicts of interest to declare.

ACKNOWLEDGMENTS

The authors would like to thank staff of the Johnstone Memorial Laboratory at Lincoln University for animal care, Ross Bland (AgResearch, NZ) for assistance with ssAAV9 packaging and purification, and Clifford Heindel at UNC for cloning the scAAV9 plasmid construct. This work was funded by CureKids NZ (to S.M.H. and D.N.P. and a post-doctoral fellowship to N.L.M) and the Batten Disease Support and Research Association (to D.N.P. and S.J.G.). N.M. was supported by a John W and Carrie McLean Trust PhD scholarship.

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