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ARTICLE

Bilateral single-site intracerebral injection of a nonpathogenic herpes simplex virus-1 vector decreases anxiogenic behavior in MPS VII mice

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Genetic diseases of the brain usually have pathologic lesions distributed throughout, thus requiring global correction. Herpes simplex virus-1 (HSV-1) vectors may be especially useful for gene delivery in these disorders since they can spread trans-synaptically along neuronal pathways to distal sites from a localized injection. We have previously shown that a nonpathogenic HSV-1 (strain 1716), which is deleted in the ICP34.5 gene, and expressing the lysosomal enzyme β-glucuronidase (GUSB) from the latency-associated transcript (LAT) promoter, spreads within the brains of GUSB-deficient mucopolysaccharidosis VII mice to reverse the pathognomonic storage lesions throughout the diseased brain. In this study, we tested the ability of the 1716 LAT-GUSB vector to improve behavioral deficits. The treatment significantly decreased anxiogenic behaviors associated with the mutation, as indicated by open-field behavior and decreased neophobia in a novel object-recognition task. The treated mice also exhibited an improvement in cognitive function associated with the cerebral cortex in a familiar object test. The results indicate the functional therapeutic potential of the 1716 LAT-GUSB vector.

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INTRODUCTION

The mucopolysaccharidoses (MPSs) are a group of 11 lysosomal storage diseases (LSDs) caused by disruptions in glycosaminoglycan catabolism, leading to their accumulation in lysosomes.[1] More than 60 LSDs have been identified, comprising ~14% of all inherited metabolic diseases affecting nearly 1:7,700 births, of which about 30% are MPSs.[1–3] The MPS subgroup of LSDs is associated with growth delay, organomegaly, cardiopulmonary disease, skeletal dysplasias, neurological dysfunction, and early death.[1]

MPS type VII, also known as Sly syndrome, was first identified in humans in 1973[4] and was found as a naturally occurring disorder in mice, dogs, and cats.[5] Mutations in the β-*glucuronidase* (GUSB) gene reduce or completely eliminate the functional enzymatic activity and lead to the accumulation of undegraded glycosaminoglycans within the lysosomes. This metabolic alteration also interferes with the expression of many other functions in cells.[6] MPS VII is a progressive condition that affects most tissues and organs and patients typically begin to show signs and symptoms during early childhood, but the severity of symptoms can vary widely among the affected individuals. The brain is a major target of pathology, and a prominent symptom of Sly syndrome is mental impairment.

A number of gene therapy approaches have been investigated in animal models to treat the central nervous system (CNS) in lysosomal diseases, including MPS VII.[7] Herpes simplex virus-1 (HSV-1) has been used as a vector to transfer and express genes in the CNS because it forms lifelong latent infections in neurons.[8] During its latent stage, HSV has only one active promoter (the latency-associated promoter (LAT)), which can be used to drive foreign genes.[9,10] Furthermore, HSV does not integrate during the latent cycle, thus there is a very low probability of insertional mutagenesis.[11] To use HSV-1 as a therapeutic vector for the nervous system, the neurovirulence gene (ICP34.5) has been deleted,[12,13] which essentially eliminates pathogenic effects even in metabolically fragile animals such as MPS VII mice.[14]

We have used an HSV-1 strain 17 mutant (1716) lacking ICP34.5 with a human GUSB cDNA driven by the LAT promoter as a vector to transfer GUSB to CNS neurons in MPS VII mice by peripheral inoculation[9,15] and direct injection into the brain.[8,16,17] A single, small bilateral injection into the striatum of adult MPS VII mice mediates widespread distribution of the vector.[18] Because this vector is replication competent, it spreads from a single injection site across several orders of neurons to effect widespread gene delivery. Injections

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into different brain structures result in various patterns of vector distribution based on connectivity to the injection site.[19] Thus the vector genome can be distributed in various ways but the subsequent secretion of the normal enzyme from transduced cells amplifies the therapeutic effect.[20] In this study, we have evaluated the effect of a single bilateral injection into the striatum, which results in GUSB expression in the cerebral cortex, diencephalon, midbrain/pons/medulla, and cerebellum,[19] on affective and cognitive function in validated mouse models of memory and anxiety, open-field behavior, interaction with familiar objects, and novel object recognition.[21–23]

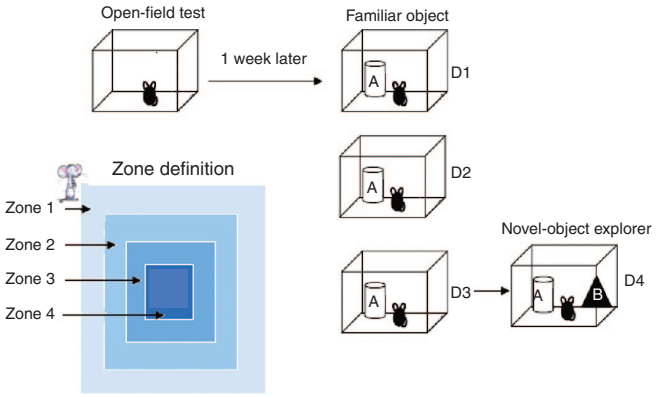

Figure 1 Experimental work flow. The mice were injected at the age of 6–8 weeks and behavior was recorded prior to injection, and 1 and 2 months postinjection. They were initially tested in the open field. One week later, familiar-object test was done for three consecutive days (D1, D2, and D3). On the following day (D4) mice were tested in the novel-object explorer test.

RESULTS

The major neurologic symptom in MPS patients is mental retardation. However, the multisystem MPS diseases also have severe bone and joint diseases, which impairs mobility, as well as severe sensory deficits. The mouse models have essentially the same spectrum of pathology as in the human diseases, thus these deficits significantly reduce their ability to perform certain behavioral tasks that are used to assess the effects of CNS.[24–26] We evaluated the ability of the HSV vector expressing LAT-GUSB to improve cognitive parameters using assays (Figure 1) that could be assessed in adult MPS VII mice with advanced disease, which includes deficiencies of hearing, sight, and mobility.

Open-field test

MPS VII mice spent more time in the perimeter (zone 1; Figure 1) compared with wild-type mice (Figure 2), an effect indicative of anxiety.[22] A two-way repeated-measures analysis of variance (ANOVA) revealed an effect of group ($F(1,10) = 8.3$, $P < 0.02$) and month ($F(2,9) = 5.6$, $P < 0.05$). There was no effect of repeated testing for wild-type mice. However, for mutant mice, the time spent in the perimeter (zone 1) increased from month 3 compared with month 1, an effect indicative of increasing anxiety ($F(2,25) = 3.6$, $P < 0.05$). The behavior of wild-type mice that were injected with 1716 LAT-GUSB into the striatum was similar to that of uninjected wild-type mice (Figure 2). Like the uninjected MPS VII mice, the MPS VII mice injected with phosphate-buffered saline (PBS) or 1716 spent more time in zone 1 at 1 and 2 months after injection ($F(2,24) = 3.9$, $P < 0.05$ and $F(2,29) = 5.7$, $P < 0.01$, respectively). In contrast, for MPS VII mice injected with 1716 LAT-GUSB, there was no effect of month of testing ($F(2,37) = 2.0$), suggesting that 1716 LAT-GUSB protects against the anxiogenic effects of the mutation. A comparison of the time spent in

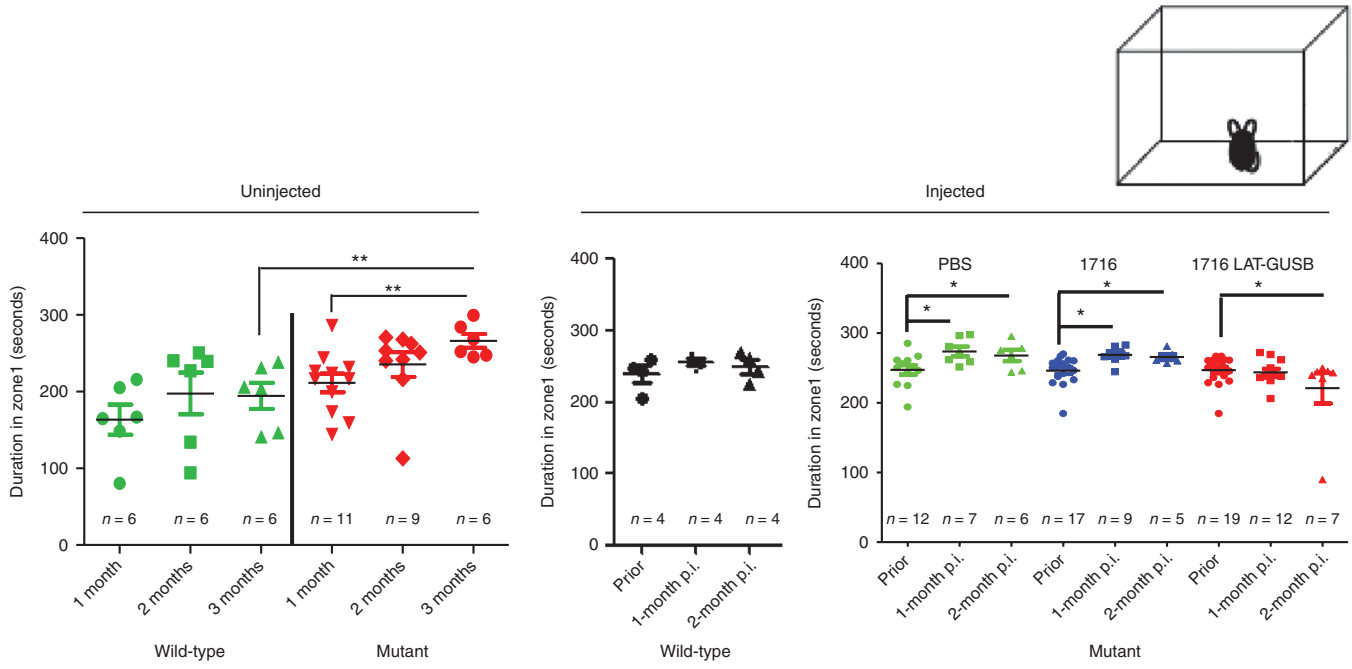

Figure 2 The 1716 LAT-GUSB virus treatment reverses the decrease in exploratory behavior seen in the mutant mice. The graphs show the duration of time spent in the periphery of the open field (zone 1) for the uninjected wild-type and mutant mice (left panel), wild-type mice injected with 1716 LAT-GUSB (center panel), and mutant mice injected with either PBS, 1716, or 1716 LAT-GUSB (right panel). Each point is the mean of at least six mice. The open-field testing of the injected mice was performed, as described in the Materials and Methods section, prior to injection (prior) and at 1 and 2 months postinjection (p.i.). Only the mutant mice injected with GUSB-expressing virus showed a significant decrease in time spent in zone 1 at 2-month p.i. The mean number of mice for each group was as follows: uninjected wild-type mice ($n = 6$), uninjected mutants ($n = 6$–11), injected wild-type ($n = 4$), PBS ($n = 6$–12), 1716 ($n = 5$–17), and 1716 LAT-GUSB ($n = 7$–19).

zone 1 between all three injected MPS VII groups showed a trend for a treatment effect ($F(2,15) = 2.7$, $P = 0.09$) and for a treatment by month interaction ($F(4,28) = 2.5$, $P = 0.06$). Particularly at 1 month after injection, the group treated with 1716 LAT-GUSB spent significantly less time in zone 1 compared with the other two groups ($P < 0.05$, Tukey HSD). The loss of statistical significance at month 2 was likely due to the decreased number of subjects as a result of mortality.

Familiar-object recognition

Exposure of wild-type mice to the same object on repeated days resulted in a decrease in time spent interacting with the object, an effect indicative of object recognition ($F(2,15) = 5.8$, $P < 0.02$) (Figure 3). Notably, the uninjected MPS VII mice had little interaction with the object, which is consistent with an anxiogenic or neophobic effect. This did not change with repeated exposure. Across months and days of testing there was a group effect ($F(1,38) = 31$, $P < 0.0001$), a group X day interaction ($F(2,37) = 9.1$, $P < 0.005$), and a group X day X month interaction ($F(4,74) = 2.7$, $P < 0.05$). The wild-type mice injected with 1716 LAT-GUSB showed a similar decrease in the duration in the interaction with the familiar object with repeated exposure prior to the injection and at 1 and 2 months after injection. There was a significant effect of day ($F(2,8) = 9.4$, $P = 0.01$), but no day X month interaction (Figure 3). Similar to the uninjected MPS VII mice, the mutant mice injected with PBS or 1716 had minimal interaction with the object, an effect indicative of anxiety, and this did not change with repeated presentations during the same month or across different months (Figure 3). However, the mutant mice injected with 1716 LAT-GUSB showed a pattern of interaction with the familiar object, which was similar to that of the wild-type mice at both 1 and 2 months after injection. Thus, they interacted with the object at 1 and 2 months after injection, indicating a lack of anxiety. Moreover, the duration of interaction decreased on subsequent days during the same month, indicating familiarity or recognition of the object. A comparison between the mutant mice injected with PBS, 1716 and 1716 LAT-GUSB revealed an effect of treatment ($F(2,79) = 12.8$, $P < 0.0001$), a treatment X month interaction ($F(4,79) = 2.9$, $P < 0.05$), a treatment X day interaction ($F(4,156) = 11$, $P < 0.0001$), and a trend toward a treatment X day X month interaction ($F(8,156) = 1.8$, $P = 0.07$). Thus, injection with 1716 LAT-GUSB reversed the anxiogenic effects associated with the object and increased recognition.

Novel-object recognition

The wild-type mice exhibited a preference for interaction with the novel object compared with a familiar object (Figure 4). In contrast, the MPS VII mutants did not exhibit a preference for the novel object, and rather the results suggested a preference for the familiar object, which is indicative of neophobia. A two-way repeated-measures ANOVA revealed an effect of group ($F(1,10) = 20$, $P < 0.005$) but no month and no group X month interaction. The mutant mice injected with PBS or 1716 showed a decrease in preference toward the novel object, and this was not seen in the MPS VII mice treated with 1716 LAT-GUSB (Figure 4). When the three mutant injected groups were compared across all three timepoints, there was a trend toward an effect of treatment ($F(2,15) = 3.0$, $P = 0.08$) and an effect of month ($F(2,14) = 4.7$, $P < 0.05$) but no month X treatment interaction ($F(4,28) = 1.9$, $P = 0.15$). However, an analysis of behavior before injection and at the 2-month timepoint showed a trend toward an effect of treatment ($F(2,15) = 3.0$, $P = 0.07$), an effect of month ($F(1,15) = 10.0$, $P < 0.01$), and a month X treatment interaction ($F(2,15) = 4.2$, $P < 0.05$). Notably, although the MPS VII

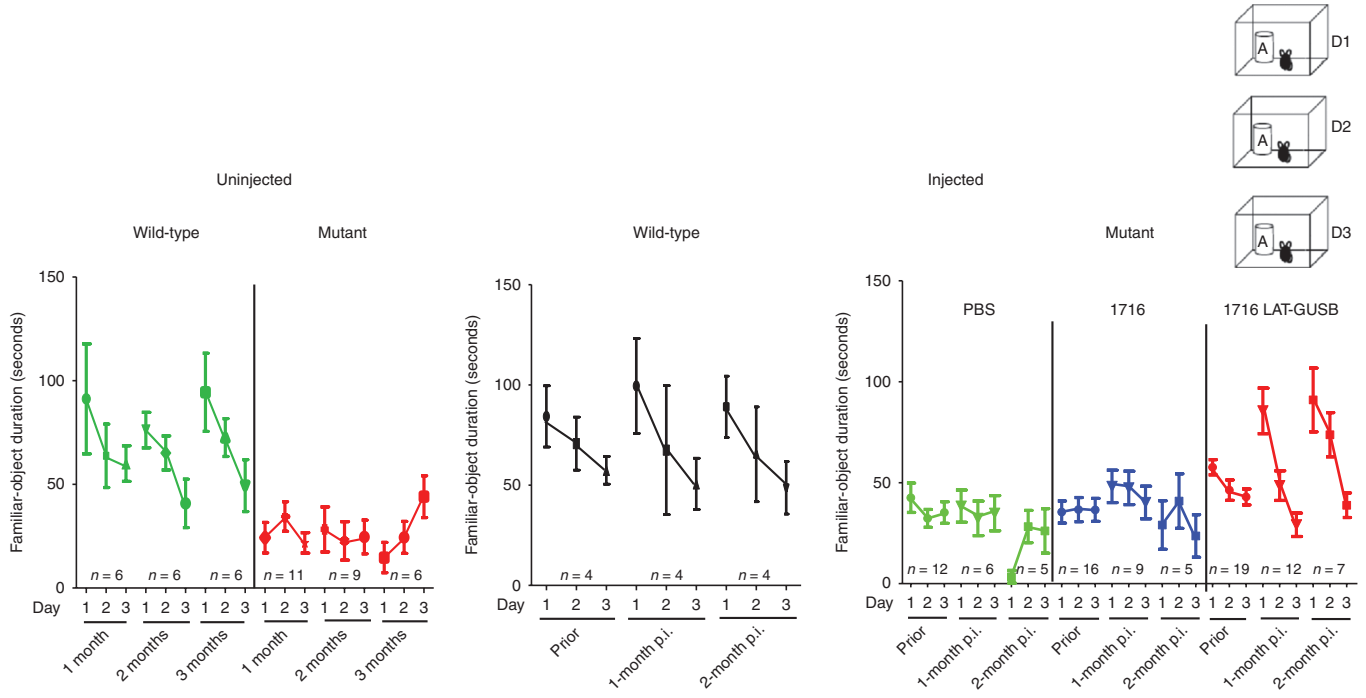

Figure 3 The 1716 LAT-GUSB virus, but not control 1716 virus or vehicle treatment, reverses impairment in the recognition of a familiar object. The graphs show the duration of time spent with the familiar object for the uninjected wild-type and mutant mice (left panel), wild-type mice injected with 1716 LAT-GUSB (center panel), and mutant mice injected with either PBS, 1716, or 1716 LAT-GUSB (right panel). The familiar-object testing of the mice was performed, as described in the Materials and Methods section, prior to injection (prior) and at 1 and 2 months post injection (p.i.). Only the mutant mice inoculated with GUSB-expressing virus showed a decrease in time spent with the familiar object at 1- or 2-month p.i.

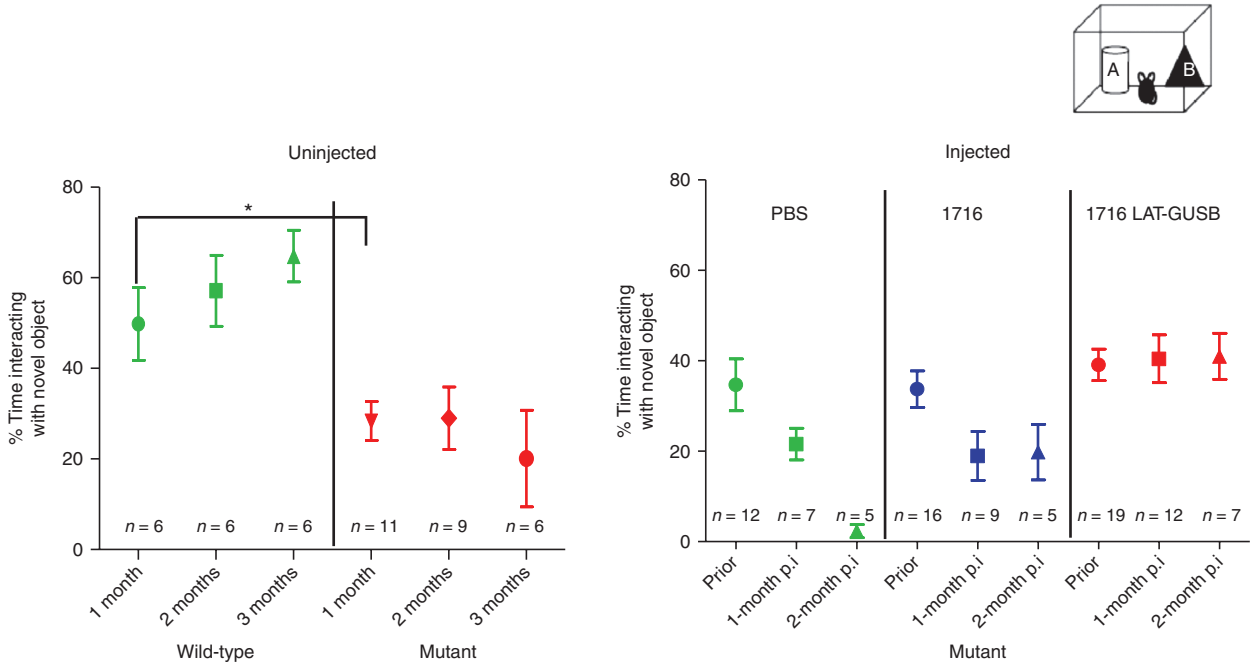

Figure 4 The HSV-1 1716 LAT-GUSB virus treatment increases the duration spent with the novel object. The graphs show the duration of time spent interacting with the novel object out of the total time spent interacting with either object for the uninjected wild-type and mutant mice (left panel), wild-type mice injected with HSV-1 1716 LAT-GUSB (center panel), and mutant mice injected with either PBS, HSV-1 1716, or HSV-1 1716 LAT-GUSB (right panel). The mutant mice and mice injected with vehicle or HSV-1 1716 showed an unusual decreased preference for the novel object, which is indicative of fear. This did not occur in the mutant mice injected with HSV-1 1716 LAT-GUSB.

mice injected with 1716 LAT-GUSB did not show a preference for the novel object as was seen in the wild-type mice, the aversion toward the novel object, which was apparent in the untreated and PBS- and 1716-treated mutants, was prevented by 1716 LAT-GUSB (Figure 4).

Post-treatment survival of mice

In order to determine the effect of 1716 LAT-GUSB on the MPS VII mice, survival data were compared using log-rank (Mantel–Cox) and Gehan–Breslow–Wilcoxon tests (Figure 5). The difference between PBS-injected and 1716-infected animals was not significant, but the mice treated with HSV 1716 LAT-GUSB lived significantly longer than the PBS-injected animals ($P < 0.05$).

GUSB enzymatic activity

The mice were sacrificed after the last behavioral assay was performed, at 2 months postinjection. Five randomly chosen brains from the treated and untreated control groups were wet-dissected into six major substructures,[6] and the midbrain segment containing the injection site (including striatum, thalamus, and hypothalamus) was homogenized and assayed for GUSB enzymatic activity by using standard methods.[27] The treated mice had significantly higher GUSB activity compared with PBS-injected MPS VII mice (Table 1) ($P < 0.01$). The level of GUSB activity in the treated MPS VII mice was 2.8% of wild-type mouse brains, which is consistent with the levels that have been shown to reverse lysosomal storage.[19,28]

DISCUSSION

This study was designed to determine whether the behavioral and cognitive impairments expressed by the MPS VII mutant mice could be reversed using the HSV-1 strain 17 virus lacking the ICP34.5 gene as a vector to express a GUSB cDNA from the LAT gene promoter. The

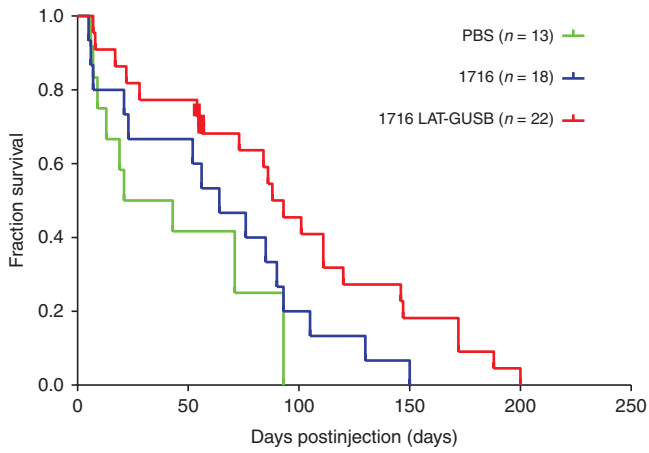

Figure 5 The survival of treated and control MPS VII mice. A Kaplan–Meier plot was constructed for each group with the green line representing PBS-injected animals, blue line representing the 1716-treated mice, and red line representing the 1716 LAT-GUSB-treated mice. Normal mice of this strain live for more than 2 years.

bilateral injections of the vector increased the brain level of GUSB in the mutant mice. The untreated mutant mice developed anxiogenic behaviors in the open-field test and in novel-object recognition, and this was greatly attenuated in the mutant mice treated with 1716 LAT-GUSB vector. Moreover, when repeatedly presented with the same object, the mutant mice exhibited neophobia that did not habituate whereas the mutant mice treated with vector showed normal interactions initially and habituation indicative of recognition. Finally, injection of the vector increased the survival time of the mutants. Together the results underscore the therapeutic utility of the HSV-1 vector to correct affective and cognitive dysfunction and enhance the life span of subjects with MPS disease.

Table 1 GUSB enzymatic activity in treated and control brains

Genotype	*Phenotype*	*Treatment*	*n*	*GUSB activity (nmol/l/mg/hr) ± SEM*	*% normal ((X – PBS)/(Norm – PBS))*
gusb +/+	Normal	None	3	54.0 ± 2.9	100
gusb –/–	MPS VII	PBS	3	1.3 ± 0.2	0
gusb –/–	MPS VII	1716 LAT-GUSB	5	2.8 ± 0.3*	2.8*

GUSB, β-glucuronidase; PBS, phosphate-buffered saline; MPS, mucopolysaccharidosis; LAT, latency-associated transcript.
*Treated versus PBS-injected MPS VII, $P < 0.01$.

The 1716 LAT-GUSB vector results in the dissemination of the GUSB gene to distal areas of the brain from the injection site.[18] The distribution varies with the injection site since the vector is transported along the synaptic pathways connected to the injection site.[19] Furthermore, studies of adeno-associated virus (AAV) vectors have shown that the enzyme can also be transported axonally to distal sites.[29,30] Thus, various injection sites can be used to achieve widespread enzyme distribution in the brain and resolve the storage lesions in the MPS VII mice.[7] To minimize the injection dose, the vector was injected bilaterally into a single site in the striatum, from where it spread to other regions of the brain.[18] Numerous studies in this model, from several laboratories, have shown that, regardless of the gene-delivery method, if the active enzyme reaches diseased cells it will reverse the lysosomal storage.[7]

A variety of behavioral tests have been used in the MPS VII disease model to determine the efficacy of various vectors to reverse the cognitive deficits associated with the disease. These include the Morris water maze for spatial learning and memory,[24,25] circadian rhythm disruptions,[31] an olfactory-based maze,[26] and auditory and novel-object recognition assays.[32] Certain dysfunctions characteristic of this disease, such as impaired mobility, deafness, and blindness due to non-neurological pathology such as bone and joint degeneration that severely affect the whole skeleton and sound conduction, and corneal opacity, can confound the interpretation of the effect of the disease on many cognitive and behavioral endpoints. Here, we selected assays to evaluate functional improvement that can be performed by a physically compromised animal. The open-field test was the only one used that involves some aspect of mobility although the endpoint itself is related more to where the animal is in the environment than magnitude of locomotion. This assay is based on the natural thigmotactic (wall-seeking) behavior of the mouse in an effort to avoid open and potentially dangerous areas.[23] This behavior is a validated assay for assessing the anxiolytic activity of drugs, which decrease the duration in the peripheral zone and increase the duration in more open spaces of the field, and anxiogenic agents, which increase thigmotaxis.[22,23] The familiar and novel object tests do not require much mobility and so are ideal for testing cognition in animals with impaired mobility. At the basis of these tests is the natural tendency of rodents to explore novel objects and to habituate once those objects become familiar. Thus, in the familiar-object test, by examining the duration spent with that object over three consecutive days, one tests the natural tendency to interact with the object when it is at first novel and when it is recognized as it is no longer novel with repeated presentations. Typically, the mice interact substantially with the object at first because it is novel and less so with repeated presentations if memory is intact. The memory impairment would be detected as less habituation. In the novel-object test, a novel object replaces the familiar one and the preference for the novel object (the duration spent with the novel object compared with the familiar object) should be greater if there is memory of what is familiar. This tests the working memory that is cortically mediated. An alternative task to the novel object is to make position novel, which tests for spatial memory and hippocampal function.[21] A previous study showed cognitive deficits in the novel-object recognition task in the MPS VII mice that were prevented by neonatal stem cell therapy when mice were treated at birth.[32]

As the mutants aged, they exhibited increased thigmotaxis in the open-field test compared with the wild-type mice, which is indicative of developing anxiety. The finding that this was not apparent in either the wild-type mice or the mutants administered 1716 LAT-GUSB indicates that this vector reverses the development of an anxiogenic phenotype. In the familiar-object test, the wild-type mice showed the characteristic behavior of interacting more with an object that is at first novel and interacting less when the object is presented on subsequent days because the object is recognized as familiar. The mutant mice showed a deficit in interactions with the object initially, which is indicative of neophobia or anxiety and consistent with the open-field test results. Additionally, there was no habituation to this response. It might be expected that if the object was recognized with repeated presentations as nonthreatening, there would be an increase in interaction. Although there was a trend toward this behavior in the untreated mutant mice, this was not significant and did not occur with mutants treated with PBS or 1716. The results suggest that the mutants exhibit both an anxiogenic phenotype and impaired memory. Notably, the mutants injected with 1716 LAT-GUSB exhibited this anxiogenic phenotype and impaired memory prior to the injection, which then reverted to the wild-type phenotype by 1 month after the injection and the phenotype was maintained at 2 months. This result underscores the enduring reversal of emotional and cognitive deficits associated with this vector. Finally in the novel-object test, the wild-type mice showed an increased preference for the novel object whereas the mutants showed a decreased preference for the novel object over time, which is indicative of neophobia and did not develop in the mice treated with the vector. Together the results of these three assays provide convergent support for the efficacy of this gene therapy in preventing the development of affective and cognitive impairments in the MPS VII mouse model.

Another notable observation is that the vector treatment reversed the cognitive dysfunctions since the treatment was initiated in adult animals that had attained a severely diseased state. This is potentially relevant for translation to humans because most LSDs are not diagnosed until the patients begin to miss developmental milestones, usually in early childhood. Although the total levels of GUSB activity in the brain may appear to be relatively low, the level of enzymatic activity that was present relative to normal brain tissue has previously been shown to result in correction.[19,28]

The finding that the mice treated with the GUSB vector lived longer suggests that treatment of the CNS component of this LSD leads to not only improved cognitive and behavioral performance but also a positive effect of correcting the CNS on the general health of the animal. We have previously reported a significant increase in life span in this disease model when treated *in utero* with an AAV vector delivered only to the brain.[33] In that study, the increased life span occurred even though there were extremely low levels of enzyme in the circulation and no improvement in skeletal dysplasia. In this study using adult animals, the CNS treatment was initiated after the disease was fully manifest, but still had a positive effect on life span, which is consistent with the interpretation that correction of the CNS may be exerting a positive effect on the overall state-of-health of the animal.

In conclusion, we have found that the intracranial injection of the 1716 LAT-GUSB vector can decrease the anxiogenic behaviors and improve the cognitive function in the mutant mice even though the treatment was started at an advanced stage of disease. Although there are significant challenges to be faced, such as scale-up in the brain size, route of administration, and an assessment of procedural safety, the benefits of HSV-mediated transduction in the CNS are potentially applicable to the treatment of a broad range of neurological disorders. In particular, the ability of a nonpathogenic HSV vector to spread to higher order neurons may allow the number of injections and amount of virus injected to be minimized relative to potential therapeutic impact. The relatively low dose that is needed for this nonpathogenic replicating vector to achieve widespread gene delivery would allow a lower dose to be injected compared with other gene-transfer vectors.

MATERIALS AND METHODS

Cells and viruses

Vero cells were grown in Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD) containing 5% fetal calf serum, 100 units/ml penicillin, and 100 units/ml streptomycin. The 1716 LAT-hGUSB vector was described previously.[18] Briefly, human GUSB gene was inserted into LAT genes under LAT promoter by homologous recombination. The vector was both grown and titered (by plaque assay) on Vero cells as previously described in ref. 34 to a titer of 3.4×10^8 pfu/ml.

Animal procedures

The MPS VII-affected (gus^{mps}/gus^{mps}) mice were produced from carrier breedings. Identification of the MPS VII-affected mice, which contain a single base-pair deletion in exon 10 of the GUSB gene, was verified by PCR genotyping, as described previously.[35] The experimental groups included 13 MPS VII mice injected with PBS, 18 MPS VII mice injected with 1716, and 20 MPS VII mice injected with 1716 LAT-hGUSB vector. To obtain a sufficient number of paired animals for the study, it was necessary to use a mixture of genders for both the control group (6 females, 12 males) and vector-GUSB group (8 females, 12 males). Multiple breeding pairs were set up at one time to generate enough affected mice, which is the limiting factor. In order to assay the large number of mutant mice used, we injected and assayed them in several groups, taking care to set up both treated and untreated mice in each of the subgroups, or mutant versus normal. The injections were done at the age of 6–8 weeks so that a group could be injected together. The behavioral assays were performed prior to injection, then at 4 and at 8 weeks postinjection. The breeding, maintenance, and experimental use of all the mice were in accordance with the guidelines of the Children's Hospital of Philadelphia Institutional Animal Care and Use Committee.

Stereotaxic intracranial injections

Injections were performed under sterile conditions. Six-to-eight-week-old MPS VII mice were anesthetized with isofluorane, secured in a stereotaxic frame (Kopf, Tujunga, CA), holes the size of the injection needle were drilled into the skull, and injections were performed bilaterally with 1 µl of PBS or 3.4×10^5 pfu virus per brain region at a rate of 0.15 µl/minute. Injections were made into the striatum (coordinates 2.00, 0.00, 3.00)[18] using a 10-µl Hamilton syringe with a 26s-gauge needle, and the needle was left in place for 3 minutes, which was then slowly withdrawn. Animals were only included in the analysis if the needle track was in the target site on postmortem histological examination. The life span calculations of significance only used animals injected at 56 d age and >10d postinjection, to exclude the acute-phase effects of HSV.

Behavioral tests

The behaviors were examined before intracerebral injection and at 1 and 2 months after injection of the same mice. The behavioral tests were done by the same investigator between 16:00 pm and 18:00 pm under standard fluorescent lighting, before the lights were turned off in a natural day–night cycle. The mice were then returned to their home cages. An open-field test (5 minutes) was performed as described by Malinowska *et al*.[36,37] The mice were placed in the center of an arena (40 cm × 40 cm × 40 cm) and the behavior was video recorded for 5 minutes and analyzed using the Noldus Ethovision XT video tracking system (Noldus Information Tech Inc, Leesburg, VA). The areas of zones 2+3+4 and zones 3+4 were ½ and 1/4 of the total field area, respectively. The duration spent in different zones of the arena, as depicted in Figure 1, was quantified. The time spent in each behavior was summed to yield an index of total activity.

The same mice that were tested in the open field were also tested in the novel-object recognition test, which evaluates nonspatial, cortically mediated learning and memory function.[21] This test was performed as described[37] with the following modifications: the mice were habituated in an open field over 1 day pre-exposure (day 1 for 5 minutes). Following 6 days of rest, an object (Figure 1, Object A; a yellow plastic dish) was placed diagonally in the open field (close to the walls) on days 7, 8, and 9, and the mice were placed in the cage and allowed to explore for 5 minutes each day. The behavior was videotaped and the time spent exploring Object A on each day was quantified. On day 10, a different object (Figure 1, Object B; a black rectangular stapler) was placed together with A, and the mice were again allowed to explore them for 5 minutes. The second object was placed at the opposite of the first object, and the mouse was placed in the center point of the field to start recording. The behavior was videotaped and the time spent exploring the objects was quantified using the Noldus Ethovision XT video tracking system. The exploration of an object is defined by the system as the nose being oriented toward the object and within a 10-mm perimeter around the object. The novel-object recognition was quantified as the duration spent exploring Object B over the total duration of exploration of both Objects A and B on day 10.

Tissue collection and enzyme assays

The mice were sacrificed after the last behavioral assay was performed, at 2 months postinjection. They were deeply anesthetized and then transcardially perfused with 10-ml cold PBS (DEPC) followed by 10-ml 4% paraformaldehyde in PBS (diethylpyrocarbonate treated). The brains were removed, put in 4% paraformaldehyde overnight, and then transferred to PBS. For quantitative enzyme assays, five randomly chosen brains from the treated and untreated control groups were wet-dissected into six major substructures, as described.[6] The untreated group contained three PBS-injected, one 1716-injected, and one uninjected brains. The three PBS-injected brains were used to calculate the significance between untreated and treated animals (Table 1) in order to use a cohort of identically manipulated animals; however, the difference was also statistically significant if the calculation was performed with all five control brains ($P < 0.02$). The piece containing the injection site, the striatum, was homogenized and assayed for GUSB enzymatic activity by using standard methods[27,28] This piece also contains the thalamus and hypothalamus, which cannot be separated accurately from the striatum in a wet dissection, and thus is homogenized and assayed as a whole subregion.[6]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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