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Intravenous injection of AAVrh10-GALC after the neonatal period in twitcher mice results in significant expression in the central and peripheral nervous systems and improvement of clinical features

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ABSTRACT

Globoid cell leukodystrophy (GLD) or Krabbe disease is an autosomal recessive disorder resulting from the defective lysosomal enzyme galactocerebrosidase (GALC). The lack of GALC enzyme leads to severe neurological symptoms. While most human patients are infants who do not survive beyond 2 years of age, older patients are also diagnosed. In addition to human patients, several naturally occurring animal models, including dog, mouse, and monkey, have also been identified. The mouse model of Krabbe disease, twitcher (twi) mouse has been used for many treatment trials including gene therapy. Using the combination of intracerebroventricular, intracerebellar, and intravenous (iv) injection of the adeno-associated virus serotype rh10 (AAVrh10) expressing mouse GALC in neonate twi mice we previously have demonstrated a significantly extended normal life and exhibition of normal behavior in treated mice. In spite of the prolonged healthy life of these treated mice and improved myelination, it is unlikely that using multiple injection sites for viral administration will be approved for treatment of human patients. In this study, we have explored the outcome of the single iv injection of viral vector at post-natal day 10 (PND10). This has resulted in increased GALC activity in the central nervous system (CNS) and high GALC activity in the peripheral nervous system (PNS). As we have shown previously, an iv injection of AAVrh10 at PND2 results in a small extension of life beyond the typical lifespan of the untreated twi mice (~40 days). In this study, we report that mice receiving a single iv injection at PND10 had no tremor and continued to gain weight until a few weeks before they died. On average, they lived 20–25 days longer than untreated mice. We anticipate that this strategy in combination with other therapeutic options may be beneficial and applicable to treatment of human patients.

1. Introduction

Krabbe disease or globoid cell leukodystrophy (GLD) is a lysosomal disorder involving the central and peripheral nervous systems (CNS, PNS). It is caused by mutations in the galactocerebrosidase (GALC) gene which lower the GALC activity resulting in the faulty catabolism of specific galactosphingolipids leading to defects in myelination (reviewed in [1,2]). While most human patients present with signs before six months of age and die before two years of age, older patients, including adults with neurologic signs, are also diagnosed [1]. The clinical course of these late-onset patients is very variable. In addition to human patients several naturally occurring animal models are available including mice, dogs and non-human primates [3–6]. These models share many pathological features with the human patients. Animal models, especially the twitcher (twi) mouse, have been used for many treatment trials. These include bone marrow transplantation (BMT) [7,8], stem cell transplantation [9–12], substrate reduction therapy [13], pharmacological chaperone therapy [14], gene therapy [15–21], enzyme replacement therapy [22,23], anti-oxidant therapy and various combinations of these treatments [18,19,24,25]. While some have resulted in prolonged life for the treated affected mice, others have been less successful. None have resulted in a complete cure.

While it is obvious that any successful treatment must supply some GALC activity, it is not known how much is needed for prevention or correction of the pathology, mainly disrupted myelination, caused by the GALC deficiency. The lack of GALC activity also results in changes in certain cytokines and chemokines [26–28]. These may also have to be corrected to result in a completely effective therapy. In addition to the obvious pathology observed in the CNS, significant pathology and

Abbreviations: GLD, Globoid cell leukodystrophy; GALC, Galactocerebrosidase; twi, Twitcher; PND, Post-natal day; CNS, Central nervous system; PNS, Peripheral nervous system; HSCT, Hematopoietic stem cell transplantation; MLD, Metachromatic leukodystrophy; LFB/PAS, Luxol-fast blue/periodic acid Schiff; PFA/PBS, Paraformaldehyde in phosphate buffered saline

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clinical features are related to defects in myelination in the PNS [29]. In fact, infant human patients treated by hematopoietic stem cell transplantation (HSCT) within five weeks of life have significant preservation of cognitive functioning, but suffer from increasing ataxia which progresses to an inability to walk within a few years of treatment [2]. It is known from studies in twi mice who received BMT alone, that they have low levels of GALC activity in the brain and sciatic nerve [7,8]. However, they do live significantly longer than untreated affected mice. It has also been shown that viral gene therapy into the brain, while prolonging the lives of the mice, does not improve all aspects of the disease and these treated mice eventually develop hind leg weakness and die shortly after that [15–21].

It is assumed that the initiation of therapy in both human and animal models as early as possible is a better strategy as it could supply some GALC activity before damage to the myelin has occurred.

Several studies have shown the ability of AAVrh10 to cross the blood brain barrier [30–32]. In studies of viral gene therapy using AAVrh10 from this laboratory [21], injection of vector intracerebroventicularly (icv), intracerebellarly (ic) and intravenously (iv) was started at PND2, shortly after genotyping of the newborn mice. This resulted in significant improvement in weight, lifespan, fertility, neuropathology and myelination. However, while these mice had prolonged life, the symptoms of hind leg weakness and paralysis eventually presented and resulted in wasting and death. When iv injections of AAVrh10 alone were done at PND2 there was a small but significant extension of life beyond the 40 days expected for untreated twi mice [21]. As it is unlikely that multiple injection sites for viral vectors will be approved for treatment of human patients, we decided to limit our viral administration to iv injection only. A later injection time, approximately PND10, was chosen for several reasons: a larger dose of virus could be given via tail vein at that age versus PND2, and PND10 corresponds to the start of major myelination in the mouse [33]. It is shown here that a single iv injection of AAVrh10-GALC at PND10-12 resulted in normal levels of GALC in the brain, very high levels in the liver, heart and muscle and much higher than normal levels in the sciatic nerve. This is an important finding indicating successful delivery of GALC activity to the PNS. The mice receiving this treatment had no tremor, a normal walking pattern, normal fertility and continued to gain weight until a few weeks before they died. Although the first mouse injected on PND10 lived 147 days until sacrificed, others lived 20–25 days longer than untreated affected mice. While the present study was meant to explore the outcome of the PND10 treatment, some results were compared with the PND2 treatment published previously [21]. These findings are critical for planning future studies to treat Krabbe disease and other disorders affecting both the CNS and the PNS.

2. Materials and methods

2.1. Generation of AAV2/rh10-mGALC vector

Construction of AAVrh10-mGALC vector was previously reported [21]. Briefly, mouse GALC cDNA was cloned into pCB7plasmid, an improved version of AAV2 vector, which was received from the Institute for Human Gene Therapy at the University of Pennsylvania. In this construct, mouse GALC cDNA is driven by the human CMV-enhancer/chicken β-actin hybrid promoter. The construct was sequenced throughout the expression cassette, and integrity of the ITRs was confirmed by restriction analysis with SmaI and NcoI. The functionality of the construct was verified by in vitro cell transfection and measurement of GALC enzyme activity. STBL2 competent cells (Life Technologies, Carlsbad, CA, USA) were used for transformation of the construct followed by carbenicillin (Invitrogen, Carlsbad, CA, USA) selection. Large-scale plasmid preparation was achieved using the Plasmid Maxi kit from Qiagen (Valencia, CA, USA). The recombinant genome was cross-packaged into AAVrh10 capsid by utilizing a chimeric AAV2-Rep/AAV1-Cap and helper plasmids during a triple-transfection procedure [34,35]. Viral packaging and purification were accomplished by the Institute for Human Gene Therapy at the University of Pennsylvania and the vector was called AAVrh10-mGALC. Viral titer, was determined by PCR of the simian virus 40 poly(A) sequence [35,36]. The viral titer of the current batch of AAVrh10-mGALC is 8.1×10^{12} genomic equivalents/ml.

2.2. Animal procedure

The original twitcher mice with W339X mutation in the C57BL background were used in this study. Homozygous twitcher (twi/twi) mice were generated by mating heterozygous animals originally obtained from the Jackson Laboratory. Affected mice obtained from heterozygous matings were genotyped on postnatal day 1 (PND1). Toe clips were used for mouse identification and DNA extraction. Genotypes were determined by polymerase chain reaction (PCR) [37] by using a sense primer, 5′-ATGAGACTGAAATTGGTAGACAGC-3′ and an antisense primer containing a single mismatch, 5′-ATGGCCCACTGTCTTCAGGTGATA-3′ to make a new recognition site for EcoRV in the mutant allele. PCR fragments were digested with EcoRV and analyzed by electrophoresis on 2.5% MetaPhor agarose gel (Lonza Inc. Allendale, NJ, USA). All injections were done on PND10-12 unless stated otherwise. Mice were observed three times per week, and, if deemed moribund, (inactive with severe twitching and weight loss), were killed by CO_2 euthanasia and the age of death was recorded. All treatments of mice were approved and carried out according to the guidelines of the Institutional Animal Care and Use Committee at Thomas Jefferson University (Protocol: 622A).

2.3. Viral delivery

Treatment strategy was a single iv injection in the tail vein of the viral vector between PND10 and 12 (this small variability in injection times was due to the timing of births). However, no differences in outcome were noted and therefore we will refer to all mice as being treated on PND10. Trypan blue was added to the viral stock at a final concentration of 0.05% (wt/vol) to facilitate visual assessment of the injections. The animals were cryo-anesthetized on ice before the injections. Injections were carried out on a light box to facilitate visualizing the tail vain. A total of 2×10^{11} viral particles in 25 μl were administered. A 28G1/2 insulin syringe was used for the injection. The needle was inserted into the vein, and the solution containing the virus was injected manually. A correct injection was verified by noting blanching of the vein. After the injection, pups were warmed up and returned to their cage. Mice that died within a few days of the injection (less than 10%) were not included in this study.

2.4. Tissue preparation for microscopic analysis

The treated and untreated mice were anesthetized with pentobarbital sodium (100 μg/g body weight) and perfused initially with 40 ml of ice-cold 1x PBS by the transcardiac route, followed by perfusion with 40 ml ice-cold 4% paraformaldehyde in phosphate buffered saline (PFA/PBS). The entire brain and cerebellum were removed from the skull, postfixed in fresh 4% PFA/PBS for an additional 4 h at room temperature, and cryoprotected by soaking in 25% glucose (*w*/*v*) for 18 to 24 h at 4 °C. Once the tissues sank to the bottom of the sucrose solution, they were embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) and frozen in a liquid nitrogen-chilled isopentane (Sigma–Aldrich, St. Louis, MO, USA) bath. The tissue blocks were stored at −80 °C until sectioning. Six-micrometer-thick coronal sections were prepared using an HM-505 N Microm cryostat (Richard-Allan, Kalamazoo, MI). Recovered sections on the glass slides were processed directly for staining or stored at −80 °C.

Alternatively, tissues from the PFA/PBS perfused mice were post fixed in 10% formalin (3.7% formaldehyde) and processed for paraffin embedding and sectioning. Two-micrometer-thick paraffin sections were prepared and stained with luxol-fast blue/periodic acid Schiff

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