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Research Report

High-throughput screening of stem cell therapy for globoid cell leukodystrophy using automated neurophenotyping of twitcher mice

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HIGHLIGHTS

- Novel biomarkers of the twitcher mouse were detected using an automated system.
- Intraperitoneal injections of stem cells were administered to twitcher mice.
- Twitcher mice receiving cell therapy had improved locomotion and motor function.
- Automated analysis of mouse behavior is effective as therapeutic screening for GLD.

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ABSTRACT

Globoid cell leukodystrophy (Krabbe's disease) is an autosomal recessive neurodegenerative disorder that results from the deficiency of galactosylceramidase, a lysosomal enzyme involved in active myelination. Due to the progressive, lethal nature of this disease and the limited treatment options available, multiple laboratories are currently exploring novel therapies using the mouse model of globoid cell leukodystrophy. In order to establish a protocol for motor function assessment of the twitcher mouse, this study tested the capability of an automated system to detect phenotypic differences across mouse genotypes and/or treatment groups. The sensitivity of this system as a screening tool for the assessment of therapeutic interventions was determined by the administration of murine bone marrow-derived stem cells into twitcher mice via intraperitoneal injection. Animal behavior was analyzed using the Noldus EthoVision XT7 software. Novel biomarkers, including abnormal locomotion (e.g., velocity, moving duration, distance traveled, turn angle) and observed behaviors (e.g., rearing activity, number of defecation boli), were established for the twitcher mouse. These parameters were monitored across all mouse groups, and the automated system detected improved locomotion in the treated twitcher mice based on the correction of angular velocity, turn angle, moving duration, and exploratory behavior, such as thigmotaxis. Further supporting these findings, the treated mice showed improved lifespan, gait, wire hang ability, twitching severity and frequency, and sciatic nerve histopathology. Taken together, these data demonstrate the utility of computer-based neurophenotyping for motor function assessment of twitcher mice and support its utility for detecting the efficacy of stem cell-based therapy for neurodegenerative disorders.

Abbreviations: GLD, globoid cell leukodystrophy; MSC, mesenchymal stromal cell; GALC, galactosylceramidase; BMT, bone marrow transplant; BMSC, bone marrow-derived multipotent stromal cell; GVHD, graft vs host disease; CNS, central nervous system; PNS, peripheral nervous system; iNOS, inducible nitric oxide synthase; GFP, green fluorescent protein; eGFPTgBMSC, enhanced green fluorescent protein transgenic BMSC; AAALAC, Association for Assessment and Accreditation of Laboratory Animal Care; IACUC, Institutional Animal Care and Usage Committee; PND, postnatal day; HBSS, Hank's Buffered Salt Solution; IMDM, Iscove's modified Dulbecco's media; ANOVA, analysis of variance; Twi, twitcher mouse; WT, normal wild-type mouse; Het, heterozygote mouse; HE, Hematoxylin and Eosin; LFB, Luxol Fast Blue; PAS, Periodic Acid-Schiff; IP, intraperitoneal.

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1. Introduction

Globoid cell leukodystrophy (GLD; Krabbe's Disease) is an autosomal recessive neurodegenerative disorder caused by deficient galactosylceramidase (GALC), a lysosomal enzyme responsible for the metabolism of glycolipids during active myelination [1–5]. A rapidly progressive disorder, GLD affects both the central and peripheral nervous systems and presents most commonly as a lethal course causing death by the age of 2 years [2,6]. The twitcher mouse model is an authentic model of human GLD that resulted from a spontaneous mutation of the GALC gene (*Galc*^{twi}) [7]. Studied mainly in the C57Bl/6 genetic background, the twitcher mouse presents at post natal day (PND) 14–15 with symptoms of motor function deterioration, weight reduction, and decreased activity. If left untreated, twitcher mice will experience rapid motor and neurological deterioration and death by PND40 [2,7,8]. Pathological features in this mouse model are similar to those in the affected human, including central and peripheral demyelination, thus highlighting the utility of these mice for studying human GLD [8–10].

Experimental treatments in the twitcher mouse have shown modest improvements in survival, although all treated animals, regardless of therapeutic approach, experience severe motor and neurological deterioration before dying prematurely [3,11–13]. The severity and progressive nature of GLD, in addition to the lack of effective treatment options, highlight the need for further development of innovative therapeutic approaches. Therapeutic studies using the twitcher mouse must aim to correct neurological and motor abnormalities in order to achieve motor function and quality of life improvement in GLD patients. Specifically, add-on therapies that target the peripheral nervous system and/or increase GALC enzyme levels are actively being tested for development of a treatment that, combined with CNS therapy, would alleviate symptoms related to widespread demyelination [14].

In order to evaluate the efficacy of new treatment combinations, measurements of motor function become necessary for comparison across treatment groups. For this purpose, many research teams have routinely used the wire hang test to assess strength in the hind legs [13,15–18], gait testing [15,19], twitcher frequency and severity scoring [15,16,20], and the rotarod test to assess balance, coordination, and strength [13,16–18]. However, there is no agreed screening protocol, and mouse groups cannot be accurately compared across laboratories due to differences in testing regimens (e.g., number of weekly tests, combination of tests performed) and scoring systems (e.g., wire hang scores defined by hang time vs. wire grasping difficulty). Additionally, the validity of observational testing is compromised by observational and analysis biases, especially when an observer is not blinded to the treatment group. Studies that provide only survivability and body weight data avoid such biases, yet the absence of motor function data decreases the translatability of a study while relying on expensive and time-consuming molecular studies to compare treatment groups that may have no effect on a patient's presentation. The goal of the present study was to apply computer-based video-tracking to detect quantitative differences in behavior and motor activity between wild-type and twitcher mice of various treatment groups. Automated neurophenotyping has become widely used to accurately monitor mouse behaviors and physiological parameters for motor function assessment [21–24], and application of this technology to the twitcher mouse model may improve sensitivity and reliability of motor function assessment and provide a means of high-throughput therapeutic screening for GLD that can be reproduced consistently in various laboratories.

2. Materials and methods

2.1. Animals

Adult (3 month old) heterozygote (Het; *Galc*$^{twi/+}$) C57Bl/6J (B6.CE-*Galc*twi/J) mice were originally obtained from The Jackson Laboratory (Bangor, ME) and used as breeder pairs to generate homozygous (Twi; *Galc*$^{twi/twi}$) twitcher mice. The established mouse colony was maintained under standard housing conditions in the pathogen-free environment of Tulane University Vivarium. Animals were housed 4–5 mice per cage with free access to food pellets and water, and the twitcher mice had access to a Diet Gel 76A nutrient fortified water gel (ClearH2O, Portland, ME) following weaning at PND 21. In total, there were six mouse groups studied: 25 untreated wild-type (WT, *Galc*$^{+/+}$), 15 BMSC treated WT (WT-BMSC, *Galc*$^{+/+}$), 13 untreated heterozygote (Het, *Galc*$^{twi/+}$), 26 untreated twitcher (Twi, *Galc*$^{twi/twi}$), 19 Hank's balanced salt solution injected twitcher (HBSS, *Galc*$^{twi/twi}$), and 13 BMSC treated twitcher (BMSC, *Galc*$^{twi/twi}$) mice were used in this study. All groups were comprised of male and female mice (Tables 1 and 2). The animals were maintained on a 12:12-h light/dark cycle (lights on at 06.00 h; off at 18.00 h), and behavioral testing was consistently conducted between 14.00 h and 18.00 h. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Tulane University and conformed to the requirements of the Animal Welfare Act. The specific *Galc* mutation was confirmed by real-time RT-polymerase chain reaction (PCR) on DNA obtained from anal swabs, as previously described [25].

2.2. Intraperitoneal injections of murine eGFPTgBMSCs

Bone marrow-derived mesenchymal stem cells (BMSCs) were obtained from male eGFP transgenic mice (C57Bl/6-Tg(UBC–GFP)30Scha/J strain; Jackson Laboratory) between 4 months and 6 months of age. BMSCs were isolated and cultured from the femurs and tibias of each mouse as previously described (Ripoll and Bunnell, 2009). Passage eight murine eGFPTgBMSCs were recovered from cryopreservation in complete Iscove's *modified Dulbecco's media* (IMDM) media (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 10% horse serum (HyClone Laboratories, Inc., South Logan, UT), 1% L-glutamine (Invitrogen), and 1% penicillin–streptomycin (Invitrogen), cultured to 70% confluency and harvested for infusion. The cells were washed thoroughly with 1×phosphate buffered saline (Invitrogen) after removing media, lifted with trypsin (Invitrogen), neutralized with an equal volume of complete media, and counted using a Countess® Automated Cell Counter (Invitrogen). The cells were centrifuged at 420 × *g* for 5 min at room temperature. After aspiration of the media, the cell pellet was resuspended at 20,000 cells/μL with Hank's balanced salt solution (HBSS, Fisher Scientific, Pittsburgh, PA) containing calcium and magnesium but no phenol red. The cells were kept on ice for no longer than one hour prior to injection. Before injection, the cell suspension was mixed thoroughly by inversion, and a 50 μL syringe with an attached 30-gauge stainless steel needle was used to uptake the necessary volume of cells. All treated animals received injections of 10 μL of the cell suspension (2×10^5 total eGFPTgBMSCs) or HBSS into the left side of the peritoneal cavity on post natal day 5 or 6 (PND 5–6). The needle was kept immobilized for 20-s (seconds) before withdrawing to avoid leakage of the cell suspension from the injection site, and the pups were then returned to their mother.

2.3. Motor function testing

Beginning on PND14, motor function tests were performed three times per week to monitor the body weight, twitching frequency and severity, wire hang, and hind stride length; all data points represent the average of scores reported by two independent observers. Twitching frequency and severity were scored using the following scoring system: Frequency–no twitching (0), rare twitching and/or mild vibration (1), intermittent (i.e., twitching more often than not but with periods of no apparent twitching) (2), constant (3); Severity–complete absence (0), fine with total head control (1), mild with decreased lateral head movement (2), moderate with no head movement (3), and severe with inability to raise head (4). The hindlimb strength and control were measured by a wire hang test, where the mice were suspended by the tail and lowered onto a horizontal wire. The scale of wire hang ranged from 0 to 4, as follows: mouse could grasp the wire using its hind legs for more than 3-s (0), grasped the wire for at least 3-s with some struggling (1), grasped the wire with its hind legs for 3-s (2), fell within 3-s (3), or fell immediately (i.e., could not grasp the wire) (4). Hind stride length was measured by applying food coloring to the paws of the mice and allowing them to walk through a tube lined with graph paper as previously described [15]. The hind stride length of both the left and right back paws were measured and averaged together by an analyst who was blinded to the genotype and treatment group. Twitcher mice were euthanized once they lost 20% of their maximum body weight or became moribund. Body weight was measured three times per week beginning on PND16 and measurements were continued until the date of euthanasia.