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Research report

β-hexosaminidase lentiviral vectors: transfer into the CNS via systemic administration

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Abstract

Brain inflammation in GM_2 gangliosidosis has been recently realized as a key factor in disease development. The aim of this study was to investigate the effects of a FIV β-hexosaminidase vector in the brain of HexB-deficient (Sandhoff disease) mice following intraperitoneal administration to pups of neonatal age. Since brain inflammation, lysosomal storage and neuromuscular dysfunction are characteristics of HexB deficiency, these parameters were employed as experimental outcomes in our study. The ability of the lentiviral vector FIV(HEX) to infect murine cells was initially demonstrated with success in normal mouse fibroblasts and human Tay-Sachs cells in vitro. Furthermore, systemic transfer of FIV(HEX) to P2 $HexB^{-/-}$ knockout pups lead to transduction of peripheral and central nervous system tissues. Specifically, β-hexosaminidase expressing cells were immunolocalized in periventricular areas of the cerebrum as well as in the cerebellar cortex. FIV(HEX) neonatal treatment resulted in reduction of GM_2 storage along with attenuation of the brain inflammation and amelioration of the attendant neuromuscular deterioration. In conclusion, these results demonstrate the effective transfer of a β-hexosaminidase lentiviral vector to the brain of Sandhoff mice and resolution of the GM_2 gangliosidosis after neonatal intraperitoneal administration.

Theme: Disorders of the nervous system
Topic: Degenerative disease: other

Keywords: β-hexosaminidase; GM2 gangliosidosis; Gene therapy; Feline immunodeficiency virus; Lysosomal storage

1. Introduction

The catabolism of GM_2 ganglioside in mammalian cells is mediated by β-hexosaminidase, a lysosomal acidic hydrolase. Structurally, human β-hexosaminidase (HEX) is comprised of two subunits, α and β, and exists in two major isoforms HEXA (α/β heterodimer) and HEXB (β/β homodimer) of which HEXA is rate limiting in human metabolism of GM_2. Patients with *HEXA* (Tay-Sachs disease) or *HexB* (Sandhoff disease) deficiency develop storage of GM_2 ganglioside in the lysosomes primarily of neurons [5]. Although HEXA is present in all cell types and tissues, neurons are characterized by a remarkably higher concentration of gangliosides than other cell types and are therefore

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highly susceptible to GM_2 lysosomal storage secondary to β-hexosaminidase deficiency, ultimately leading to cellular dysfunction and neurodegeneration [11,33,41,42]. Affected patients present with neurodegeneration, mental and motor deterioration, muscular flaccidity, blindness, dysarthria, impaired thermal sensitivity, increasing dementia and the characteristic macular cherry-red spots. In the mouse, two genes also encode for β-hexosaminidase [37]. Due to species variation in GM_2 metabolism, targeted deletion of the murine $HexB^{-/-}$ locus is required for the development of GM_2 storage in mice [28,36,38].

Recent studies on the pathophysiology of GM_2 gangliosidosis revealed the presence of activated microglia and macrophages in the brain of $HexB^{-/-}$ knockout mice, along with increased levels of several inflammation-related genes [12,24,40]. It was also suggested that glial activation and brain inflammation contributes to neurodegeneration as it appeared to precede temporally and spatially neuronal cell death [24,40]. To this end, transplantation of healthy bone marrow to $HexB^{-/-}$ pups attenuated the microglia/macrophage activation, reduced the extent of GM_2 storage and ameliorated the clinical phenotype [25]. The aforementioned studies suggest a critical role of brain inflammation in the pathogenesis of GM_2 gangliosidosis.

Previous studies on the development of β-hexosaminidase adenoviral vectors demonstrated restoration of HEXA activity in cells in vitro [1,19] as well as in HexA-deficient mice in vivo [10]. The aim of this study was to investigate whether systemic administration of a lentiviral vector would result in the transfer of the bicistronic transgene HEXB–IRES–HEXA, encoding for both isoforms of the human enzyme [17], to the brain of HexB-deficient (Sandhoff disease) mice. Neonatal administration was elected on the basis that stable transduction of host cells early in postnatal development would lead to timely β-hexosaminidase restoration and ultimately to disease prevention. Furthermore, the incomplete state of the blood–brain barrier (BBB) of a neonate along with the neonatal inability to elicit satisfactory immunologic response to various antigenic challenges further supported our treatment strategy. Since brain inflammation, lysosomal storage and motor dysfunction are characteristics of HexB deficiency and GM_2 gangliosidosis, these parameters were employed as experimental outcomes in our study.

2. Materials and methods

2.1. Development of viral vectors

Construction of the bicistronic transgene HEXB–IRES–HEXA encoding both subunits of the human β-hexosaminidase (pHEX) has been previously described [17]. pHEX was transiently expressed in the hamster embryonic kidney BHK-21 cell line (CCL-10; American Tissue Culture Collection, Manassas, VA) following transfection by the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) per manufacturer's instructions. Human HEXA and HEXB expression was subsequently assessed at the mRNA, protein and enzyme activity levels.

The defective, VSV-G pseudotyped, FIV vector CTRZLb, named FIV(lacZ) herein, has been previously described by Poeschla et al. [29] and was kindly provided to us by Dr. David Looney (University of California at San Diego) along with the pseudotyping [3] and packaging plasmids. A Nhe I-Not I segment containing the bicistronic β-hexosaminidase gene was cloned in the place of lacZ in the CTRZLb vector (SstII-Not I) by blunt cohesive ligation to generate the FIV(HEX) transfer vector. FIV vectors were packaged in 293-H cells, as previously described [18]. In brief, T75 flasks were seeded with 293-H cells which were grown to subconfluency in DMEM plus 10%; FBS (Gemini, Woodland, CA). The cells were then cotransfected with the transfer vector, pFIV(HEX), the packaging [29] and the VSV-G pseudotyping vectors [3] using the Lipofectamine 2000 reagent (Invitrogen) per manufacturer's instructions. Twenty-four hours after transfection, the supernatant medium was discarded and replaced by fresh medium. Sixty hours after transfection, the virus-rich supernatant was collected, filtered through .45 mm Surfil®-MF filter (Corning Seperations Division, Acton MA) and subsequently concentrated by overnight centrifugation at 7000 *g* using a Sorvall RC5B high-speed centrifuge and a SLA-3000 rotor. Subsequently, the supernatant was decanted, and the viral pellet was resuspended overnight in 1 mL of normal buffered saline containing 40 mg/mL lactose at 4 °C. The viral solution was then aliquoted and frozen (−80 °C) until further use. Titering was performed on CrfK cells (American Tissue Culture Collection, Manassas, VA) cultured in 24 well tissue culture plates. Titers were calculated at 10^8 infectious particles/mL for FIV(HEX) by X-HEX [17] histochemistry. The effectiveness of FIV(HEX) to transduce murine cells was initially tested in primary murine fibroblasts (donated by Dr. M. Kerry O'Banion, University of Rochester). Moreover, FIV(HEX) efficacy was evaluated using human fibroblasts derived from a healthy volunteer and a patient suffering from Tay-Sachs disease (TSD); these cells were purchased from the Coriell Institute for Medical Research (cat. No. GM11853; Camden, NJ). Cultured normal and TSD human cells in serum-free medium (OptiMem; Invitrogen) were challenged by the exogenous administration of GM2 ganglioside (Sigma, St. Louis, MO) at the dose of 1 mg/mL for 24 h followed by a fresh OptiMem media change and infection by FIV(HEX) at approximately m.o.i. ~2. Forty-eight hours later, the cells were fixed with 4% paraformaldehyde and studied under light microscopy.

2.2. Animal genotyping

$HexB^{+/-}$ knockout breeder pairs on pure 129/sv background [27] (kindly provided by Dr. Richard Proia, Genetics

Division, NIDDK/NIH, Bethesda, MD) were mated to produce homozygous HexB$^{-/-}$ knockout mice at a 0.25 expectancy ratio. Genotyping was performed by PCR of DNA extracts from tail biopsies employing the following primer sets: $^{5'}$ATT TTA AAA TTC AGG CCT CGA$^{3'}$, $^{5'}$CAT AGC GTT GGC TAC CCG TGA$^{3'}$ and $^{5'}$CAT TCT GCA GCG GTG CAC GGC$^{3'}$. The latter were allowed to grow to maturity (60 days old) and were than employed as breeders to deliver HexB$^{-/-}$ pups at a 1.00 expectancy ratio for the subsequent experiments.

2.3. Animal injections

All protocols employing laboratory animals were reviewed and approved by the University Committee on Animal Resources. HexB$^{-/-}$ knockout mice were injected intraperitoneally (N=3) with 10^7 infectious FIV(HEX) particles in 100 μL of normal saline. An equal number of HexB$^{-/-}$ knockout mice (N=3) received saline treatment and served as controls. This group was sacrificed 5 weeks after treatment, and the expression of the β-hexosaminidase transgene was evaluated in the brain, liver and spleen by immunocytochemistry. In a second experiment, HexB$^{-/-}$ mice (N=5) were administered 10^7 infectious particles FIV(HEX), and an equal number of pups (N=5) received 100 μl of saline intraperitoneally at postnatal day P2; these mice were then sacrificed at 12 weeks of age. Moreover, wild-type littermates were employed as controls and were also sacrificed at 12 weeks of age. In a third experiment, five (N=5) HexB$^{-/-}$ mice were administered 5×10^6 infectious particles FIV(HEX), and five additional HexB$^{-/-}$ knockout pups (N=5) received 5×10^6 infectious particles FIV(lacZ) in a total volume of 100 μl intraperitoneally at postnatal day P2; these mice were sacrificed at 16 weeks of age.

Additional HexB$^{+/-}$ (N=5) as well as wild-type mice (N=5) were included in the study as controls.

2.4. PCR and RT–PCR

The tissue samples were rinsed with sterile 0.15 M phosphate-buffered saline pH 7.2 (PBS), and total RNA/DNA extracts were collected and precipitated utilizing the TRIzol reagent per manufacturer's instructions (Invitrogen). RNA samples were reconstituted in DEPC-treated ddH_2O, and 260/280 nm readings were spectrophotometrically obtained. A total of 2 μg RNA was treated with DNase I (Invitrogen) for complete destruction of deoxyribonucleotides per manufacturer's instructions followed by reverse transcription reaction employing the First cDNA Strand Synthesis kit per manufacturer's instructions (Invitrogen). RT conditions included reactions with and without the presence of the reverse transcriptase enzyme. Subsequently, a semiquantitative polymerase chain (PCR) reaction was performed employing the Platinum Taq DNA polymerase (Invitrogen) with PCR primers specifically designed for the HEXB cDNA: $^{5'}$AGT CCT GCC AGA ATT TGA TAC C $^{3'}$ and $^{5'}$ATT CCA CGT TCG ACC ATC C$^{3'}$ (Ta=58 °C) [17]. Specifically, a master mix of PCR reagents including buffer, Mg^{2+}, dNTPs and Taq polymerase was prepared. A standard curve of HEXB cDNA was prepared based on 1:2 serial dilutions of an initial 1 ng/μl stock. The samples, unknowns and standards, were then amplified by PCR for 35 cycles (within linear amplification). PCR products were then analyzed by agarose gel electrophoresis and stained with ethidium bromide, images of which were captured using an EDAS Imaging analysis system (Kodak, Rochester, NY). Samples included RT(+) and RT(−) products, pHEX vector DNA (100 nM) that served as positive PCR control, as well as primers control (no template). The standard curve data were plotted and analyzed by regression analysis. The standard curve was employed in translating the differences in band intensity measured on the gel to cDNA levels. We adapted the same protocol for IL-1 β ($^{5'}$GAGAACCAG-GAGAACCAGCAACGACAAAATACC$^{3'}$ and $^{5'}$GCATTA-GAAACAGTCCAGCCCATAC$^{3'}$), TNFα ($^{5'}$CGAGTGAC-AAGCCTGTAGCC$^{3'}$ and $^{5'}$GGTTGACTTTCTCCTG-GTATGAG$^{3'}$), IL-6 ($^{5'}$ATGTTCTCTGGGAAATCGTG$^{3'}$ and $^{5'}$GAAGGACTCTGGCTTTGTCTT$^{3'}$) and ICAM-1 ($^{5'}$CAGTCGTCCGCTTCCGCTAC$^{3'}$ and $^{5'}$AGAAAT-AGAAATTGGCTCCGTGGTCCC$^{3'}$) transcript levels, as previously described [14].

2.5. X-gal, X-HEX and fast garnet histochemistry

Tissue sections processed by X-gal histochemistry were washed in 0.15 M phosphate-buffered saline (PBS) containing 0.05% Triton-X (pH 7.2) for 60 min, followed by X-gal staining, as previously described [18]. The tissue was then washed in 0.15 M PBS for 30 min, briefly rinsed with dH_2O and counterstained with nuclear Fast Red. For histochemical detection of β-hexosaminidase, the aforementioned protocol was modified by employing 5-bromo-4-chloro-3-indonyl N-acetyl-β-D-glucosaminide (Sigma) [27]. Fast Garnet histochemistry evaluates total β-hexosaminidase activity on fixed cells or tissues following incubation of fixed cells or tissue with a solution containing 1 mg/ml Napthol AS-BI N-acetyl-B-glucosaminide in 0.1 M citrate/trisodium citrate buffer with 10% ethylene glycol monomethyl ether plus 0.5% $CaCl_2$ and 1% polyvinyl alcohol for 2 h at 37 °C. The supernatant is then discarded and the cells or tissue thoroughly washed with 0.1 M acetate buffer. The enzymatic reaction is then visualized by Fast Garnet incubation (1 mg/ml in 0.1 M acetate buffer pH 6.2) at room temperature for 30 min (red-colored stain), washed with fresh PBS and temporarily stored at 4 °C. Tissue slides can be cover-slipped using a PBS/glycerol based mounting media (Supermount; Bio-Genex). The tissue sections were studied under light microscopy using a B×51 Olympus microscope (Tokyo, Japan), and microphotographic images were captured in TIFF 16-bit format using a SPOT RT Color CCD digital

camera attached onto the microscope and connected to a DELL computer.

2.6. Enzyme detection assays

Total β-hexosaminidase activity (HEXA and HEXB) was evaluated after incubation with 4-methylumbelliferone glucuronide (4MUG), whereas HEXA activity was selectively assessed by 4 MUG-6-SO_4 (4MUGS). In brief, brain homogenates were sonicated in 20 mM Tris–HCl/137 mM NaCl/1 mM $MgCl_2$/1 mM $CaCl_2$/1 mM vanadate/1 mM aprotinin/0.5 mM PMSF buffer with 1% NP-40 for 20 s using a Branson Sonifier 450 with attached microtip and dispensed in 250 μl aliquots, which are then combined with equal volume of 2× sodium citrate/phosphate reaction buffer (pH 4.4) containing 2 mM of 4MUG or 4MUGS and incubated for 1 h at 37 °C. In addition, a standard curve of 1:2 dilution series of wild-type brain homogenate 4MUG and 4MUGS activity was determined semiquantitatively by assessing the fluorescent product of the substrate–enzyme reaction using a Packard Instruments Fluorometer at 565 nm wave length. Total protein concentration was evaluated as described above. Results were normalized to total protein levels and expressed as percent relative to wild-type tissue.

2.7. Immunocytochemistry

For immunocytochemical detection of antigens in the brain, we adapted methods, as previously described [15,16]. In brief, for the detection of β-hexosaminidase, we employed a goat polyclonal IgG antibody [32] raised against human HEXB protein (1:1000) kindly provided by Dr. Richard Proia (Genetics Division, NIDDK/NIH, Bethesda, MD). Activated astrocytes were identified by a mouse antiglial fibrillary acidic protein (GFAP) monoclonal antibody (1:400 dilution; Chemicon INTL, Temecula, CA; clone GA-5). Activated denditric cells/microglia/macrophages were stained with a rat antimajor histocompatibility complex class-II (MHC-II; Bachem, Torrance, CA; clone ER-TR3). GM_2 ganglioside was immunolocalized employing a mouse anti-*N*-acetyl GM_2 monoclonal IgM antibody (Seikagaku, East Falmouth, MA; clone MK1-16). These antibodies were coupled with appropriate secondary antibodies: rabbit antigoat IgG biotin-conjugated, goat antimouse IgG Fab biotin conjugated, goat antirat IgG biotin-conjugated antibodies and goat antimouse IgM biotin conjugated, respectively (Jackson ImmunoResearch, West Grove, PA).Visualization was performed utilizing DAB (3,3′ diaminobenzidine)-nickel as chromagen. The glass slides were then dehydrated through multiple ethanol solutions, cleared through xylene and cover-slipped using DPX permanent mounting medium (Fluka, Neu-Ulm, Switzerland). The tissue sections were then studied under a B×51 Olympus light microscope and color microphotographic images were captured as described above. The total number of $GFAP^+$ and $MHC\text{-}II^+$ cells were counted in 10 random microscopic fields (40×) as follows. In each field, the number of positive cells were counted, and averages and standard deviations were calculated for each area of the brain.

2.8. Behavioral evaluation

Behavioral assessment was evaluated by the inverted mesh method and the rotorod apparatus, as previously described [36]. The inverted mesh method evaluates neuromuscular condition by assessing grip strength. In brief, we constructed a clear plastic cylinder (20×20×30 cm) that was covered on the one end by a wire mesh. The mesh wire bars were 1 mm in diameter and 1 cm apart. A rectangular area of the screen was taped so that the animals were confined in the center of the mesh. After the mice were placed on the screen, the cylinder was turned up side down over bedding; the lapse time until their fall from the mesh was recorded in seconds. If a mouse fell in less than 10 s in the first try, this animal was given a second chance. The total lapse time until the mouse fell off the mesh was recorded. The rotorod appliance (Columbus Instruments, Columbus, OH) is comprised of a rotating cylinder spinning at a constant 10 RPM; the mice were placed on the rotating cylinder, and the total lapse time until each mouse fell off was recorded.

3. Results

The expression of both subunits of the human β-hexosaminidase from our bicistronic gene HEXB–IRES–HEXA was assessed in vitro after transient expression of pHEX in the hamster BHK-21 cell line. HEXA and HEXB expression was demonstrated at the mRNA level by RT–PCR (Fig. 1A), enzyme activity level by 4MUG/4MUGS fluorometry (Fig. 1B), protein level by immunocytochemistry (Fig. 1C–F), as well as activity level by Fast Garnet histochemistry (Fig. 2G–H).

The ability of the β-hexosaminidase lentiviral FIV(HEX) vector to transduce murine cells was initially determined in normal mouse and subsequently in human Tay-Sachs fibroblasts. Murine wild-type fibroblasts were infected with FIV(HEX) at m.o.i.~1, and β-hexosaminidase expression was found increased compared to FIV(lacZ) infected cells as assessed by X-HEX histochemistry (Fig. 2A–B). Transgene incorporation was confirmed by PCR (Fig. 2C), and gene expression was evaluated at the transcription level by RT–PCR (Fig. 2D). Moreover, Tay-Sachs cultured primary fibroblasts were treated with FIV(HEX) at m.o.i.~2, 24 h after being challenged with GM_2 (1 mg/mL) under serum-free conditions. FIV(HEX) administration to GM_2-challenged Tay-Sachs fibroblasts resulted in rescue of their spindle-like morphology and allowed their proliferation in vitro as soon as 2 days following FIV treatment (Fig. 2E–G). The fact that a greater number of wild-type fibroblasts

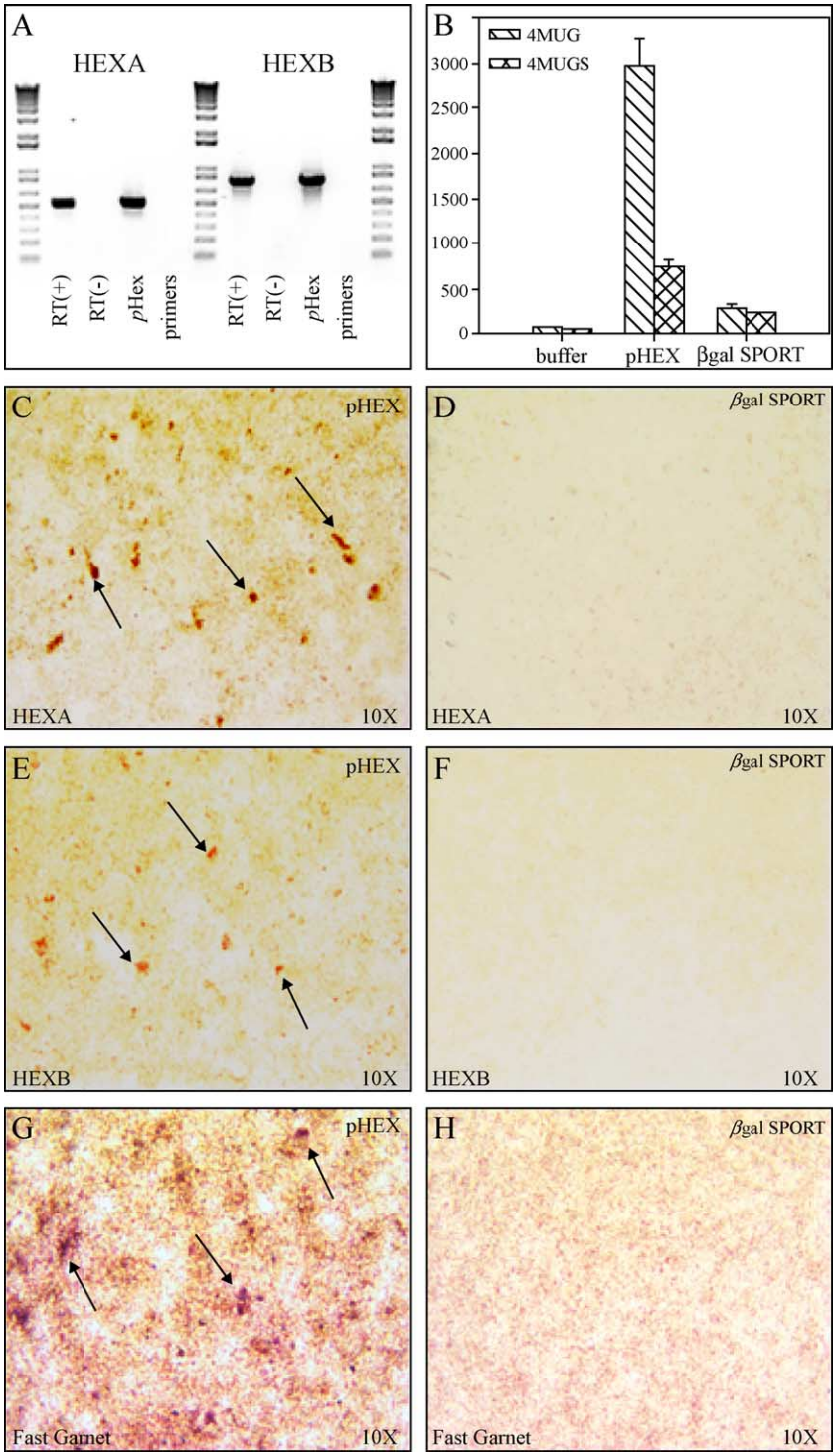

Fig. 1. The bicistronic HEXB–IRES–HEXA gene expresses both subunits of the human β-hexosaminidase. Hamster embryonic BHK-21 cells were transduced with the pHex plasmid (HEXB–IRES–HEXA) in vitro, and the expression of the human HEXA and HEXB subunits was evaluated at the mRNA, protein and enzyme activity levels. (A) HEXB and HEXA transcript was assessed in total RNA brain extracts by RT–PCR using primers that selectively anneal to the human but not the hamster cDNAs. (B) Enzyme activity levels for the HEXA and HEXB subunits were evaluated by 4 MUGS (HEXA only) and 4 MUG (HEXA+HEXB) activity. (C) The human HEXA (α/β) and (E) HEXB (β/β) β-hexosaminidase isoforms were detected in the aforementioned cells by immunocytochemistry employing antibodies that selectively detect the human proteins. Panels (D and F) depict HEXA and HEXB immunocytochemistry of cells transfected by the control vector βgalSPORT. (G) Total β-hexosaminidase activity was detected by the Fast Garnet histochemical method in cells transfected with pHex plasmid compared to cells transfected with the control vector βgalSPORT (H).

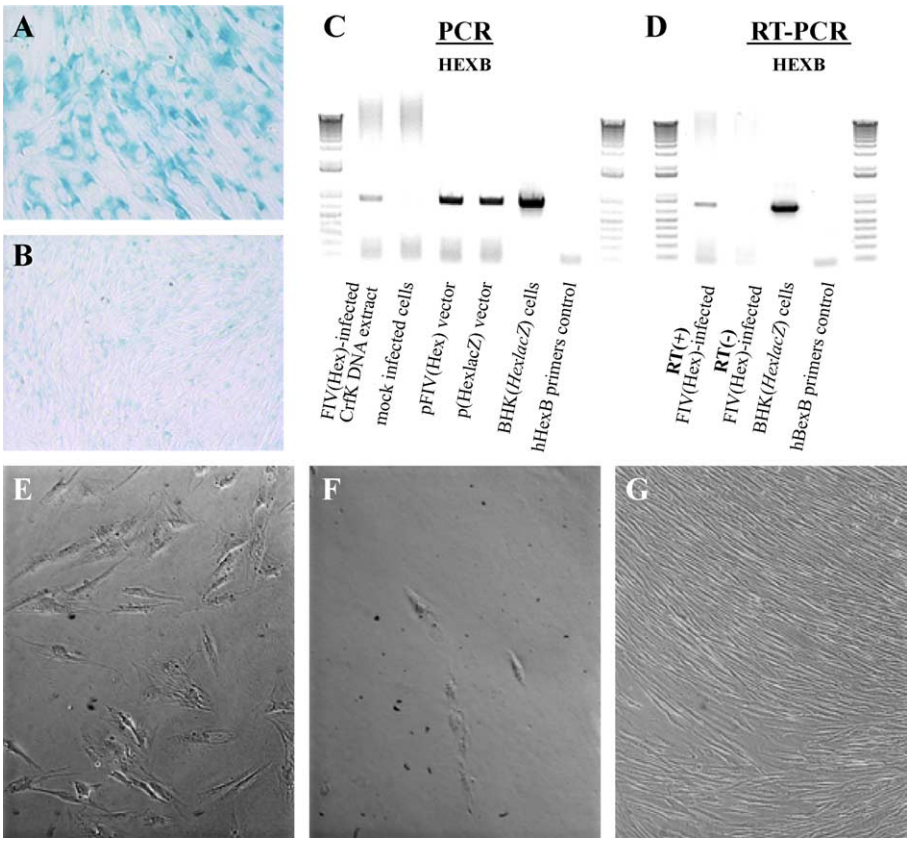

Fig. 2. The recombinant β-hexosaminidase feline immunodeficiency virus vector FIV(HEX) successfully transduced normal murine and human Tay-Sachs fibroblasts in vitro. (A) Murine wild-type primary fibroblasts were infected with FIV(HEX) at m.o.i.~1.0 in vitro, and β-hexosaminidase expression was found increased compared to (B) FIV(lacZ)-infected cells by means of X-Hex histochemistry. (C) The presence and (D) expression of the β-hexosaminidase transgene was determined in FIV(HEX)-infected murine normal fibroblasts by PCR and RT–PCR, respectively. In addition, (E) FIV(HEX) treatment conferred survival of human primary fibroblasts derived from a Tay-Sachs patient that were challenged by exogenous administration of GM_2 ganglioside, which otherwise induces cell death (F) under serum-free conditions in vitro. (G) In contrast, normal human fibroblasts were not affected by GM_2 administration under the same conditions.

resisted the exogenous GM2 challenge compared to TSD cells indicates that FIV(HEX) transduction efficacy of defective cells is less than healthy wild type cells suggesting that lysosomal storage–GM2 challenge possibly imposes a functional deficit to cells.

FIV(HEX) was also tested in vivo by injecting $HexB^{-/-}$ pups at postnatal day P2 intraperitoneally (single dose of 10^7 infectious particles) and evaluating β-hexosaminidase expression by HEXB immunocytochemistry. In the liver (Fig. 3A), transduced cells were primarily localized surrounding the portal triads (Fig. 3B). In the brain, β-hexosaminidase positive cells were located in periventricular areas of the cerebrum (Fig. 3C), which histologically appeared as ependymal as well as glial cells. Moreover, β-hexosaminidase expression was also localized in cerebellar cortical cells that appeared as Purkinje neurons (Fig. 3D). HEXB transgene mRNA expression was also evaluated, along with a number of inflammation-related genes, in the brain of $HexB^{-/-}$ mice treated with FIV(HEX), as well as in wild-type littermates that served as controls (Fig. 4A). FIV(HEX) treatment of $HexB^{-/-}$ pups resulted in detectable expression of HEXB mRNA in the brain at levels approximately 21% of that observed in wild-type littermates. Transduction with FIV(HEX) resulted in reduction of IL-1β mRNA levels compared to $HexB^{-/-}$ mice treated with saline. In contrast, TNFα was elevated following FIV(HEX) administration compared to saline-treated knockout mice and wild-type littermates. Moreover, FIV(HEX) treatment of $HEXB^{-/-}$ knockout mice restored HEXB enzyme activity level at approximately 22% of that seen in wild-type mice (Fig 4B). Overall, FIV(HEX) treatment resulted in partial restoration of β-hexosaminidase activity, attenuation of IL-1β ($p<0.05$) and, interestingly, further exacerbation of TNFα expression in the brain ($p<0.05$).

The degree of microglia and astrocyte activation was employed as an additional measure of brain inflammation and was assessed by MHC-II and GFAP immunocytochemistry, respectively. As anticipated, we found significant numbers of GFAP-positive astrocytes in the brain of 3-month-old $HexB^{-/-}$ mice in the thalamus (Fig. 5A), as well as basal ganglia and cerebellum. FIV(HEX)-treated $HexB^{-/-}$ mice showed reduced numbers of GFAP-positive cells in all the aforementioned areas of the brain (Fig. 5B), which were higher than the immunostaining in control mice

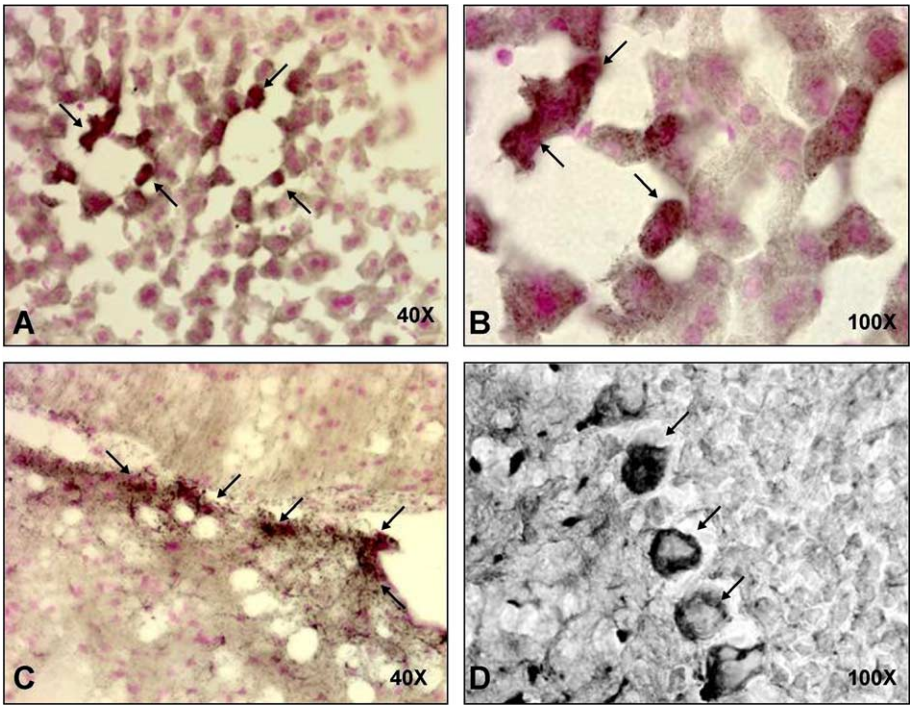

Fig. 3. Neonatal FIV(HEX) intraperitoneal administration to HexB$^{-/-}$ pups results in transduction of brain and peripheral cells. HEXB protein was detected by immunocytochemistry in (A) the liver of 5-week-old HexB$^{-/-}$ mice treated systemically with FIV(HEX) at postnatal day P2. HEXB expression was observed primarily at the portal triads. (B) Larger magnification of panel (A). (C) HEXB-positive cells were also immunolocalized in the cerebral parenchyma adjacent to the third ventricle, as well as (D) in Purkinje-like cortical cerebellar cells.

(Fig. 5C). Comparable brain sections revealed lack of MHC-II staining, suggesting the absence of activated microglia/macrophages/monocytes in all experimental groups (Fig. 5D–F). Moreover, we examined the degree of GM_2 storage in comparable brain sections by immunocytochemistry (Fig. 5G–L) and found reduced levels of GM_2 immunostaining in the brain stem (Fig. 5G), hippocampus (Fig. 6H) and thalamus (Fig. 5I) of HexB$^{-/-}$ mice treated with FIV(HEX) compared to saline-treated animals (Fig. 5J–L). Heterozygous HexB$^{+/-}$, as well as wild-type mice, did not display any positive GM_2 immunostaining (data not shown).

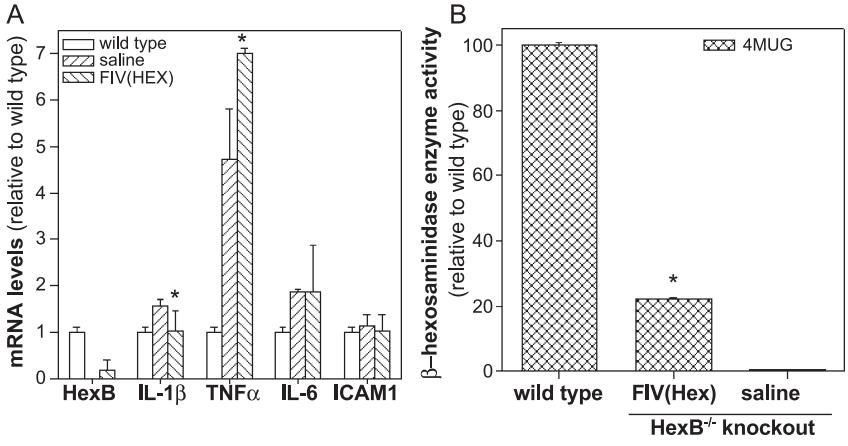

Fig. 4. HEXB expression was restored in the brain of Sandhoff mice following neonatal FIV(HEX) administration. Two-day-old (P2) HexB$^{-/-}$ pups (N=5) received a single dose (10^7 infectious particles) of FIV(HEX) intraperitoneally. At 3 months of age, the animals were sacrificed, and the mRNA levels of HexB as well as a number of inflammation-related genes were assessed by RT–PCR. Human and murine HEXB expression was detected concomitantly at the mRNA level in the FIV(HEX)-treated mice and calculated as approximately 21% of the wild-type littermates. As expected, saline-treated HexB$^{-/-}$ knockout mice did not display any β-hexosaminidase activity. In addition, IL-1 β and ICAM-1 mRNA levels normalized in the brain of HexB$^{-/-}$ mice after FIV(HEX) intraperitoneal injection, IL-6 collectively showed no overall change, whereas TNFα was found increased in the FIV-injected mice. (B) HEXB subunit activity in the brain was assessed by 4MUG fluorometry in HexB$^{-/-}$ knockout mice treated with FIV(HEX) or saline and were compared to wild-type animals. β-hexosaminidase activity was restored in the FIV(HEX)-treated mice at approximately 22% of the wild-type levels. *p<0.05.

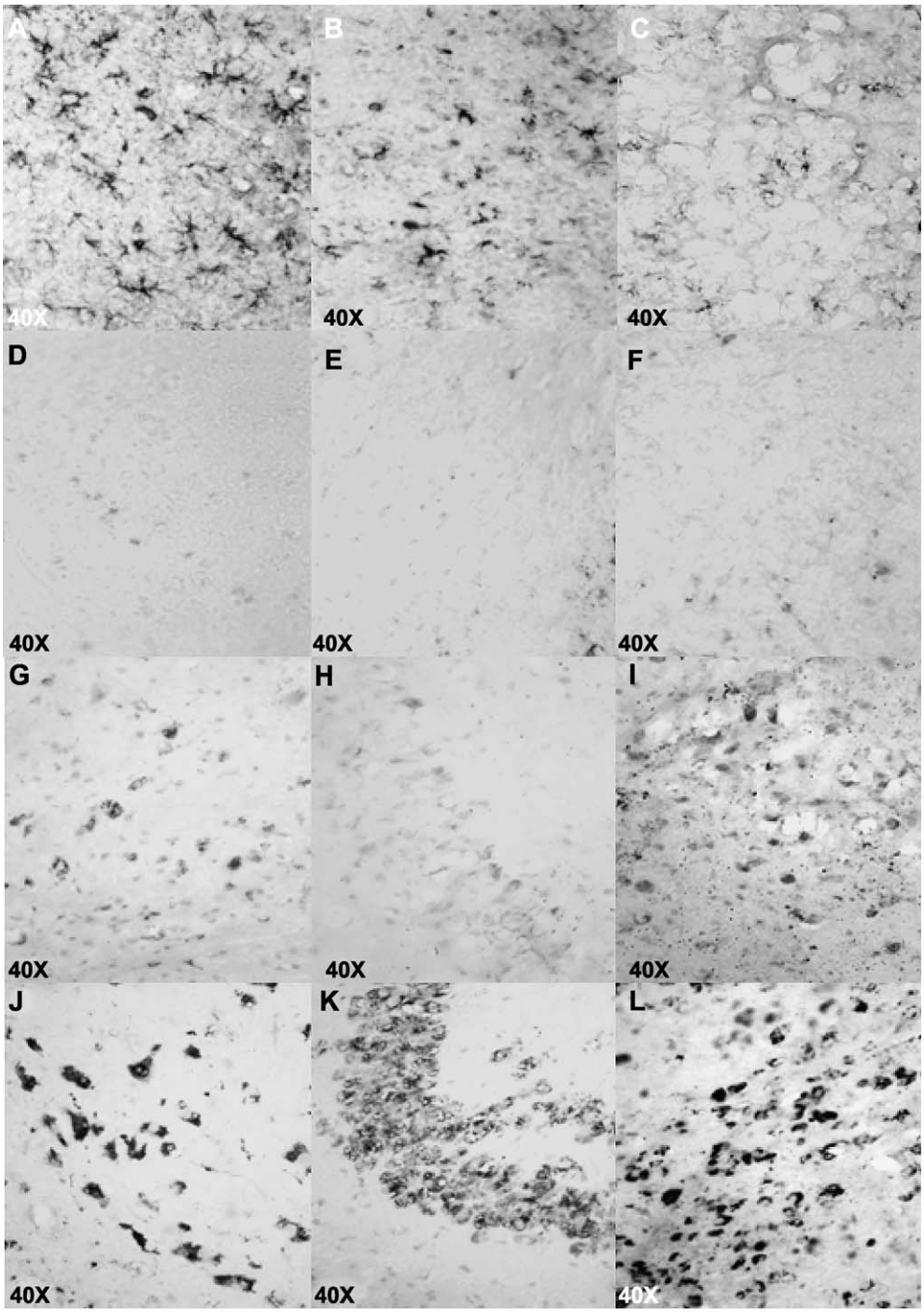

Fig. 5. FIV(HEX) neonatal administration attenuated neuroinflammation, GM2 storage and prevented cell loss in $HexB^{-/-}$ mice. Two-day-old (P2) $HexB^{-/-}$ mice (N=5) received a single dose (10^7 infectious particles) of FIV(HEX) intraperitoneally and were subsequently sacrificed at 3 months of age. Histologic brain sections were analyzed by immunocytochemistry employing antibodies against glial fibrillary acidic protein (GFAP), major histocompatibility complex-II (MHC-II) and GM_2 ganglioside. GFAP immunostaining was found increased in the thalamus of (A) $HexB^{-/-}$ mice compared to (B) FIV(HEX)-treated animals and (C) wild types. MHC-II immunostaining (thalamus) showed no evident differences between the groups (D–F). GM_2 immunostaining was also decreased in $HexB^{-/-}$ animals after FIV(HEX) treatment compared to saline-injected mice in the brain stem (panels [G] vs. [J]), hippocampus (panels [H] vs. [K]), as well as thalamus (panels [I] vs. [L]).

Similarly, the numbers of MHC-II and GFAP-positive cells were found reduced in FIV(HEX)-treated $HexB^{-/-}$ mice 4 months after treatment compared to FIV(lacZ)-treated animals (Table 1), whereas wild-type animals lacked any MHC-II or GFAP-positive cells in the brain (data not shown). Specifically, GFAP immunostaining was decreased in the cerebellum, thalamus, cortex, brain stem and basal ganglia of $HexB^{-/-}$ mice treated with FIV(HEX) compared

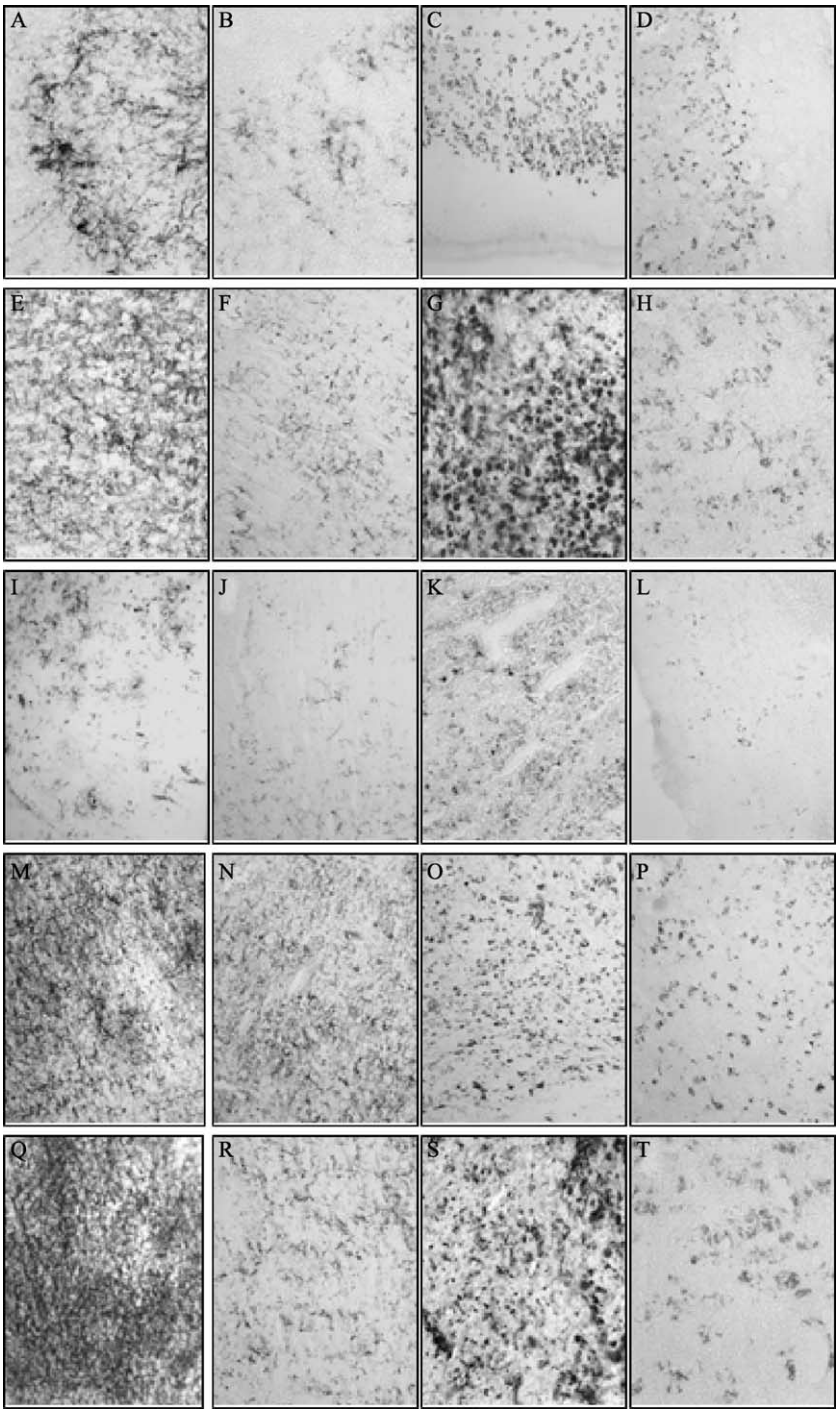

Fig. 6. FIV(HEX) neonatal administration attenuated neuroinflammation, GM2 storage and prevented cell loss in HexB$^{-/-}$ mice. Two-day-old (P2) HexB$^{-/-}$ mice (N=6) received a single dose (5×10^6 infectious particles) of FIV(HEX) or FIV(lacZ) intraperitoneally. At 4 months of age, all mice were sacrificed. Astroglial and microglial activation was evaluated by immunocytochemistry employing antibodies raised against GFAP and MHC-II antigens, respectively. FIV(lacZ)-injected animals displayed numerous GFAP- and MHC-II positive cells in the cerebellum (A and C, respectively), thalamus (E and G, respectively), cortex (I and K, respectively), brain stem (M and O, respectively) and the basal ganglia (Q and S, respectively). In contrast, FIV(HEX)-treated animals showed reduced levels of GFAP and MHC-II immunostaining in the cerebellum (B and D, respectively), thalamus (F and H, respectively), cortex (J and L, respectively), brain stem (N and P, respectively) and the basal ganglia (R and T, respectively).

to FIV(lacZ)-treated littermates (Fig. 6). MHC-II immunostaining was also decreased in all the aforementioned areas of the brain following FIV(HEX) treatment (Fig. 6). In addition, FIV(HEX) neonatal administration resulted in amelioration of the neuromuscular deficiency expressed as decreased grip strength seen in HexB$^{-/-}$ mice at 4 months of age (Fig. 7) as well as locomotive behavior as assessed by the rotorod method (Fig. 8) without any significant effects

Table 1
The number of $GFAP^+$ and $MHC\text{-}II^+$ positive cells were counted in 10 random microscopic fields (40×) in the cortex, basal ganglia, thalamus, cerebellum and brain stem of 4-month-old FIV(HEX)- and FIV(lacZ)-treated mice

		Cortex	Basal ganglia	Thalamus	Cerebellum	Brain stem
GFAP	FIV (lacZ)	35±7	293±45	80±10	52±12	225±15
	FIV (HEX)	10±4	40±8	10±5	5±3	85±12
MHC-II	FIV (lacZ)	27±5	125±25	148±32	80±14	118±12
	FIV (HEX)	7±4	35±12	30±7	45±9	45±7

In each field, the number of positive cells was counted, and average ± standard deviation was calculated for each area of the brain.

on the overall development of the mice, as assessed by total weight (data not shown).

4. Discussion

The purpose of this study was to investigate the effects of a defective, VSV-G pseudotyped, β-hexosaminidase FIV vector in the brain of HexB-deficient (Sandhoff disease) mice following intraperitoneal administration to pups of neonatal age. Since brain inflammation, lysosomal storage and neuromuscular dysfunction are characteristics of HexB deficiency, these parameters were employed as experimental outcomes. Systemic neonatal administration of FIV(HEX) resulted in restoration of HEXB in the brain of affected mice, leading to reduction of brain inflammation, GM_2 storage and cell death along with amelioration of neuromuscular dysfunction.

It was not until recently that brain inflammation was realized as an important feature of GM_2 gangliosidosis [12,24,27]. It was also suggested that the aforementioned brain inflammation contributes to neurodegeneration; the presence of activated microglia and/or macrophages in the brain preceded neuronal cell death and were observed proximal to neurons undergoing apoptosis. In support is the study by Norflus et al. [25] that described attenuation of brain inflammation and amelioration of the disease phenotype following normal bone marrow transplantation to $HexB^{-/-}$ pups. Recent studies from our laboratory on systemic FIV vector administration suggested transduction of peripheral immune cells and subsequent infiltration of peripheral blood mononuclear cells (monocytes/macrophages) into the brain parenchyma [17]. In fact, the ability of genetically modified peripheral blood mononuclear cells to enter and engraft into the brain has been previously described [30,31]. Therefore, we hypothesized that systemic

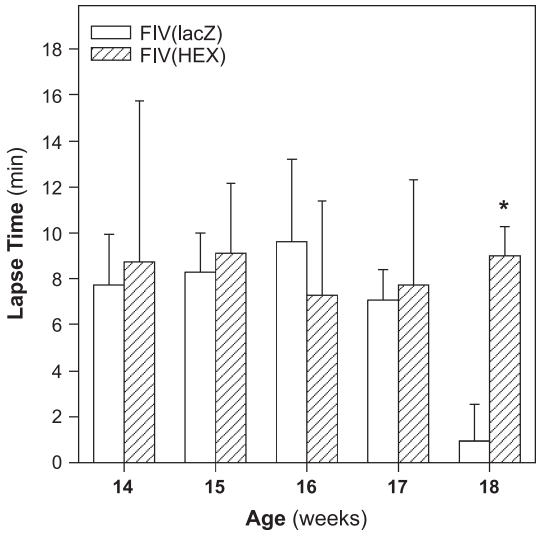

Fig. 7. FIV(HEX) neonatal administration ameliorated neuromuscular grip strength in $HexB^{-/-}$ knockout mice. Two-day-old (P2) $HexB^{-/-}$ mice (N=6) received a single dose (5×10^6 infectious particles) of FIV(HEX) or FIV(lacZ) intraperitoneally. From 12 to 16 weeks of age, the mice were evaluated for loss of grip strength by the inverted mesh method, at which time point all mice were sacrificed due to Animal Welfare regulations because two of the FIV(lacZ)-injected animals displayed complete locomotive deterioration. The FIV(HEX)-treated animals showed significantly improved locomotive performance compared to FIV(lacZ)-injected mice (p=0.00248).

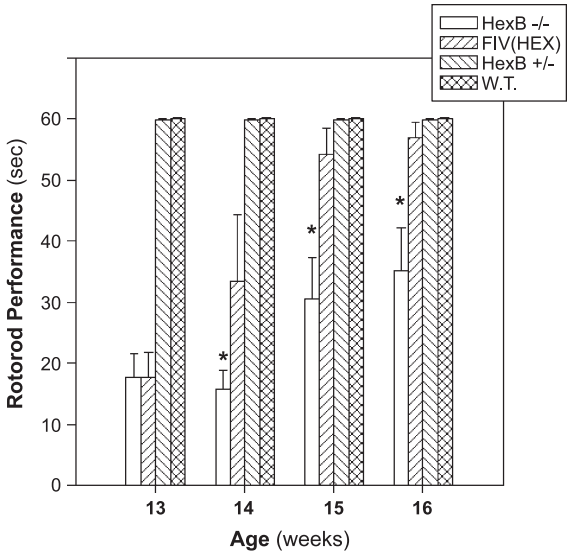

Fig. 8. FIV(HEX) neonatal administration improved locomotive performance in $HexB^{-/-}$ knockout mice. Two-day-old (P2) $HexB^{-/-}$ mice (N=5) received a single dose (5×10^6 infectious particles) of FIV(HEX). Additional $HexB^{-/-}$ mice (N=5) received saline IP injections and served as controls. Locomotive performance was evaluated by the rotorod method from 14 to 17 weeks of age. The FIV(HEX)-treated animals showed significantly improved locomotive performance compared to mock-treated mice and showed no difference compared to wild-type and $HexB^{+/-}$ hetorozygotes (p=0.0006).

FIV(HEX) administration to HexB$^{-/-}$neonatal mice would successfully transduce peripheral immune cells with the β-hexosaminidase transgene, which in turn would infiltrate into the brain and possibly result in cross-correction of the attendant β-hexosaminidase deficiency.

Initially, the effectiveness of neonatal FIV administration to infect brain and peripheral tissues was evaluated by examining the expression of the reporter gene β-galactosidase over time in wild-type mice treated intraperitoneally with FIV(lacZ) at postnatal day P2. Our results demonstrated the presence of β-galactosidase positive cells in the brain, spleen and liver, the expression of which increased with time, suggesting stable transduction of precursor cells by FIV(lacZ). Previous investigations on the effectiveness of perinatal systemic administration as the basis for global gene therapy have demonstrated detectable transgene expression for several months after the initial injection that varied between the tissues examined [20,22,40]. In our experiment, the majority of the X-gal positive brain cells appeared to be microglia, monocytes, macrophages and/or endothelial cells based on their localization and histologic appearance. In fact, previous work in our laboratory demonstrated that FIV(lacZ) intraperitoneal administration results in transduction of CD31-(endothelial cells), CD3-(lymphocytes) and CD11b-(monocytes/macrophages) positive cells by means of double immunofluorescence [18]. Similarly, Daly et al. [6] reported widespread distribution of a β-glucuronidase transgene in a mouse model of Sly disease following perinatal systemic administration of an adeno-associated viral vector. Moreover, in a subsequent study, the authors reported that neonatal gene transfer of β-glucuronidase viral vectors lead to long-term attenuation of mucopolysaccharidosis along with amelioration of the aberrant phenotype in the Sly disease mouse [8].

In the present study, administration of FIV(HEX) to HexB$^{-/-}$ pups also resulted in transduction of brain cells that were primarily localized in periventricular areas and to a lesser extent in perivascular areas of the cerebrum. A previous study demonstrated that β-hexosaminidase-bearing cells were mostly detected in the leptomeninges and choroid plexus and to a lesser extent in perivascular areas of the brain and spinal cord after normal bone transplantation to HEXB$^{-/-}$ pups [27]. This periventricular pattern of β-hexosaminidase expression in HexB$^{-/-}$ brain after FIV(HEX) treatment is somewhat different from that observed in wild-type mice following FIV(lacZ) administration, both in terms of location as well as number of cells. It is possible that these differences are a result of abnormal immune function in the HexB$^{-/-}$ mice, whereby cellular immunity and the monocyte–macrophage system in particular are impaired by the β-hexosaminidase deficiency. In fact, Kieseier et al. [13] reported that the monocyte–macrophage system, in addition to T- and B-lymphocytes, is affected in a number of storage human disorders. Moreover, abnormal immune function has been described in human patients suffering from Sly disease [7] as well as Gaucher disease [4]. Therefore, possible immune system anomalies in GM_2 gangliosidosis may result in impaired response to FIV(HEX) administration, leading to decreased numbers of transduced immune cells.

Two potential mechanisms may account for the presence of transduced cells in the brain parenchyma: direct transduction and/or trafficking of transduced peripheral cells into the CNS. In the first scenario, FIV(HEX) may successfully enter into the CNS and directly infect resident cells, such as microglia and/or astrocytes. The blood–brain barrier (BBB) is a structure unique to the central nervous system and is the result of tight junctions between the brain endothelial cells [9]. Previous work on the development of mouse BBB using large protein molecules (horseradish peroxidase) suggested BBB formation during the late days of embryonic life (E17 in mouse) [34]. Furthermore, BBB in the adult is not absolute, whereby certain areas of the brain do not develop BBB and thus allow for free exchange of molecules through them. These areas include the median eminence (hypothalamus), pituitary, choroids plexus, pineal gland, subfornical organ, organum vasculosum lamina terminalis and area posterma [35]. Hence, one could visualize the intrusion of virions into the brain matter through an incomplete BBB, as well as through areas lacking BBB during the first few days after birth. Our data demonstrate transduction of periventricular brain cells with the β-hexosaminidase transgene, as well as cerebellar cortical Purkinje-like cells. These results are supported by previous studies whereby transduction of brain immune cells and Purkinje neurons were observed following systemic administration of a β-galactosidase FIV vector [18]. In this latter study, however, it was suggested that transduction and infiltration of peripheral immune cells into the brain most probably accounts for the presence transduced cells in the brain parenchyma following cell engraftment [30], cell fusion with Purkinje cells [43] or neurogenesis [31]. Systemic administration of β-hexosaminidase FIV vectors to HexB$^{-/-}$ pups may in fact transduce hematopoietic precursors, which in turn give rise to β-hexosaminidase expressing peripheral immune cells. Although all these aforementioned mechanisms may well account for the presence of β-hexosaminidase positive cells in the brain of FIV(HEX)-treated HexB$^{-/-}$ mice, the reduction of GM_2 storage, the attenuation of brain inflammation along with the amelioration of the clinical pathology after neonatal treatment are suggestive of effective cross-correction of the underlying β-hexosaminidase deficiency.

Receptor-mediated enzyme transfer (cross-correction) is an important characteristic of lysosomal enzymes, including β-hexosaminidase, whereby secreted enzyme can be uptaken by neighboring cells via paracrine pathways. The transport and compartmentalization of soluble lysosomal enzymes to lysosomes depend on the recognition of mannose 6-phosphate (Man-6-P) residues in their oligosaccharide moiety by specific receptors. Two distinct proteins have been thus far identified capable of interacting with lysosomal enzymes, the Man-6-P receptor (MPR; 270 kDa)

which also binds the insulin-like growth factor-II (IGF-II) and the cation-dependent MPR (CD-MPR; 46 kDa) [23]. To this end, previous studies demonstrated that neural cell lines stably expressing HEXA when transplanted into normal fetal and newborn brains of mice resulted in significant levels of active hexosaminidase protein throughout the engrafted brain [19]. Moreover, unilateral intracerebral injection of an FIV vector encoding for the human β-glucuronidase gene in adult Sly disease mice resulted in bihemispheric reduction of lysosomal storage and attenuation of the clinical pathology [2]. Collectively, these studies indicate that successful transduction of a subset of brain cells with a therapeutic gene can lead to lysosomal storage resolution through the brain parenchyma even at areas distant to the site of transgene expression.

In conclusion, the data presented herein suggests that neonatal intraperitoneal administration of FIV(HEX) to $HexB^{-/-}$ pups resulted in transduction of brain cells with the therapeutic β-hexosaminidase gene, leading to reduction in neuroinflammation, attenuation of the GM_2 storage and amelioration of the attendant neurodegeneration and motor behavioral deterioration. The applicability of our findings may also extend beyond the specific lysosomal storage disorder since brain inflammation has been considered as a contributing factor in other neurodegenerative brain disorders as well [21,26,39].

Acknowledgments

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