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Short communication

The trigeminal retrograde transfer pathway in the treatment of neurodegeneration

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ABSTRACT

The trigeminal sensory system was evaluated for the retrograde transfer of gene therapy vectors into the CNS. The feline immunodeficiency viral vector, FIV(HEXB), encoding for the human HEXB gene, was injected intra-articularly in the temporomandibular joint of 12 week-old $HexB^{-/-}$ mice displaying clinical and histopathological signs of Sandhoff disease. This treatment regiment reduced GM_2 storage and ameliorated neuroinflammation in the brain of $HexB^{-/-}$ mice, as well as attenuated behavioral deficits. In conclusion, retrograde transfer along trigeminal sensory nerves may prove to be a valuable route of gene therapy administration for the treatment of lysosomal storage disorders and other neurodegenerative diseases.

1. Introduction

The lack of effective treatment for lysosomal storage disorders, including Tay–Sachs and Sandhoff disease (GM_2 storage due to β-hexosaminidase deficiency), prompts the development of new treatments. Available therapies with variable efficacy are enzyme replacement therapy and bone marrow transplantation. Although these therapies address the lack of metabolic activity in peripheral tissues, they only partially avert disease progression in the central nervous system due to the presence of the blood-brain-barrier. Gene therapy has also been tested in animal models as a potential treatment of β-hexosaminidase deficiency (Akli et al., 1996; Lacorazza et al., 1996). In a recent paper by Cachon-Gonzalez et al. (2006), intracranial injection of a recombinant adeno-associated viral vector encoding for HexA and HexB in HexB−/− neonates resulted in amelioration of GM2 gangliosidosis and attenuation of the clinical phenotype. Recent studies in our laboratory employing feline immunodeficiency (FIV) and human immunodeficiency viral (HIV) vectors for the transfer of a β-hexosaminidase therapeutic gene to HexB−/− mouse pups at neonatal stages of development demonstrated significant restoration of β-hexosaminidase activity in the brain accompanied by a reduction in the levels of GM2 storage (Kyrkanides et al., 2005, 2007a). However, the overall therapeutic efficacy was reduced by the elicitation of host inflammatory responses secondary to viral vector antigens, administered either systemically (Kyrkanides et al., 2005, 2007a) or by intracranial injection (Olschowka et al., 2003).

As we consider the development of new therapies for lysosomal storage disorders, we aim at (1) restoring enzyme activity in the CNS while (2) minimizing host inflammatory reactions, and (3) employing routes of administration that bypass the blood brain barrier. The goal of the present study was to evaluate the trigeminal retrograde transfer pathway as a novel route of gene therapy administration for the treatment of GM2 gangliosidosis and neurodegeneration, as previous studies showed efficient transfer of FIV vectors from temporomandibular joint sensory afferents to brain stem nuclei (Kyrkanides et al., 2005). Moreover, intra-articular injections of FIV vectors into the immunologically privileged temporomandibular joint, do not elicit any significant host inflammatory response (Kyrkanides et al, 2007b).

2. Materials and methods

The human HEXB cDNA (ATCC; Manassas VA), was cloned into the FIV vector (Systems Biosciences; Mountain View, CA) between Xba I–Sal I, forming the FIV(HEX) vector. FIV(HEX) was packaged and tittered as previously described (Kyrkanides et al., 2005).

All animal procedures were pre-approved by the Institutional Animal Care and Use Committee and assigned protocol number 2003-142. Breeding and genotyping of $HexB^{-/-}$ mice has been previously described (Kyrkanides et al., 2005). A total of 42 mice were employed in this study: 7 $HexB^{-/-}$ and 5 $HexB^{+/-}$ mice with FIV(HEX) treatment, as well as 10

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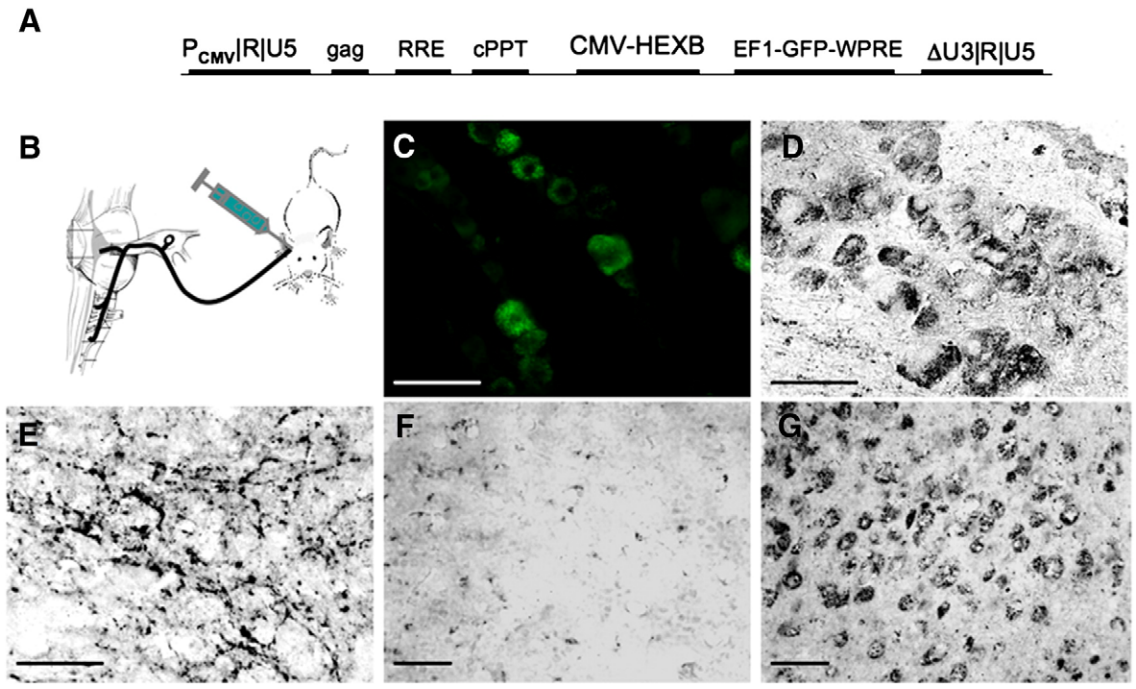

Fig. 1. Trigeminal retrograde FIV(HEX) transfer from the temporomandibular joint ameliorates GM_2 storage in cortical neurons. (A) The recombinant feline immunodeficiency virus FIV(HEX) encoding for the human β-hexosaminidase HEXB gene (a total of 10^5 infectious particles in 50 μL) was in injected (B) intra-articularly in the temporomandibular joint of adult (12 week old) $HexB^{-/-}$ knockout mice. The mice were sacrificed at 18 weeks of age. (C) Trigeminal sensory neurons were transduced by the FIV vector, as demonstrated by the expression of the reporter gene gfp (green immunofluorescence), which is expressed off the vector backbone, in trigeminal neurons' cell bodies located in the trigeminal ganglion. Furthermore, (D) the expression of the human HEXB protein in sensory neurons in the trigeminal ganglion was detected by immunohistochemistry. (E) Human HEXB protein was also detected in nerve fibers located in the trigeminal principal sensory nucleus at the dorsal medulla. (F) GM_2 storage was ameliorated in the cortex of $HexB^{-/-}$ mice following administration of FIV(HEX) compared to (F) administration of the control vector FIV(gfp) as evaluated by immunohistochemistry. Bar = 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$HexB^{-/-}$, 10 $HexB^{+/-}$ and 10 wild type littermates without any treatment, which served as controls. Anesthetized (40 mg/kg ketamine IP) $HexB^{-/-}$ mice were injected with 50 μl containing 1×10^6 FIV(HEX) infectious particles in the right and left TMJ at 12 weeks of age. Mouse behavior was evaluated weekly by rotarod performance (Columbus Instruments, Columbus OH). The rotarod appliance evaluates an animal's ability to stay on a rotating cylinder (10 rpm) and measures the lapse time that the animal can run on the rotating cylinder. Mice suffering from neurodegeneration, such as β-hexosaminidase deficiency, display reduced rotarod performance (Sango et al., 1995). The mice were subsequently sacrificed at 18 weeks of age.

The brain tissue was dissected and frozen in −80 °C until ready for analysis. Total RNA was isolated using Trizol reagent from whole brains (Invitrogen, Carlsbad, CA). Details on first strand DNA synthesis and analysis by real-time quantitative polymerase chain reaction for the genes IL-1β, TNFα, MHC-II, GFAP and COX-2 were conducted as previously described (Kyrkanides et al., 2008). In addition, human HEXB protein was detected by immunohistochemistry employing a goat anti-HEXB polyclonal antibody (Proia et al., 1984). Activated astrocytes were identified by a rabbit anti-GFAP (Dako USA; Carpinteria, CA); activated microglia/macrophages were stained with a rat anti-MHC II antibody (Bachem, Torrance, CA; clone ER-TR3); for $CD45^+$ detection we utilized a rat anti-$CD45^+$ monoclonal antibody (Serotec; Raleigh, NC; Cat. No. MCA43G); GM_2 ganglioside was immunolocalized employing a mouse anti-N-acetyl GM_2 monoclonal IgM antibody (Seikagaku, East Falmouth, MA; clone MK1-16). For more details on immunohistochemistry please refer to Kyrkanides et al. (2008). The total numbers of $GFAP^+$, $MHC\text{-}II^+$, TNFα and $CD45^+$ cells were counted in 10 random microscopic fields of each section (40×) covering the whole brain at a 240 μm resolution: positive cells were counted in each field, and averages and standard errors of mean were calculated for each area of the brain.

2.1. Statistical analysis

Differences between groups were evaluated by one-way analysis of variance (ANOVA) with alpha = 0.05 and power equal to 0.8. Post-hoc analysis was performed by the Tukey method.

3. Results

FIV(HEX) was in injected intra-articularly (10^5 infectious particles) in the right and left TMJ of 12 week-old $HexB^{-/-}$ mice (Fig. 1A–B). FIV uptake by trigeminal sensory neurons, retrograde transfer and expression in the CNS was detected by HEXB immunohistochemistry (Fig. 1C). Retrograde transfer of FIV vectors was further demonstrated by the concurrent detection of the reporter gene gfp in cell bodies located in the trigeminal ganglia (Fig. 1D) expressed off the FIV backbone.

The level of GM2 storage was reduced in cortical neurons and throughout the brain parenchyma as evaluated by immunohistochemistry (Fig. 1E). Conversely, untreated $HexB^{-/-}$ littermates displayed widespread GM2 neuronal storage (Fig. 1F). Rotarod performance, a clinical measure of disease progression, was ameliorated as a result of treatment (Fig. 2A). There were no differences in weight gain/loss between the animal groups (Fig. 2B).

Another cardinal characteristic of GM2 gangliosidosis is the development of brain inflammation. To this end, untreated $HexB^{-/-}$ mice displayed increased levels of IL-1β, TNFα, MHC-II, GFAP and COX-2 expression compared to wild type littermates. Conversely, FIV(HEX)-treated $HexB^{-/-}$ mice demonstrated amelioration of some (IL-1β and TNFα), but not all inflammatory genes (Fig. 2C).

An additional measure of brain inflammation employed in our studies was the assessment of reactive glial cells in brain. As anticipated, $HexB^{-/-}$ mice were characterized by significantly higher numbers of

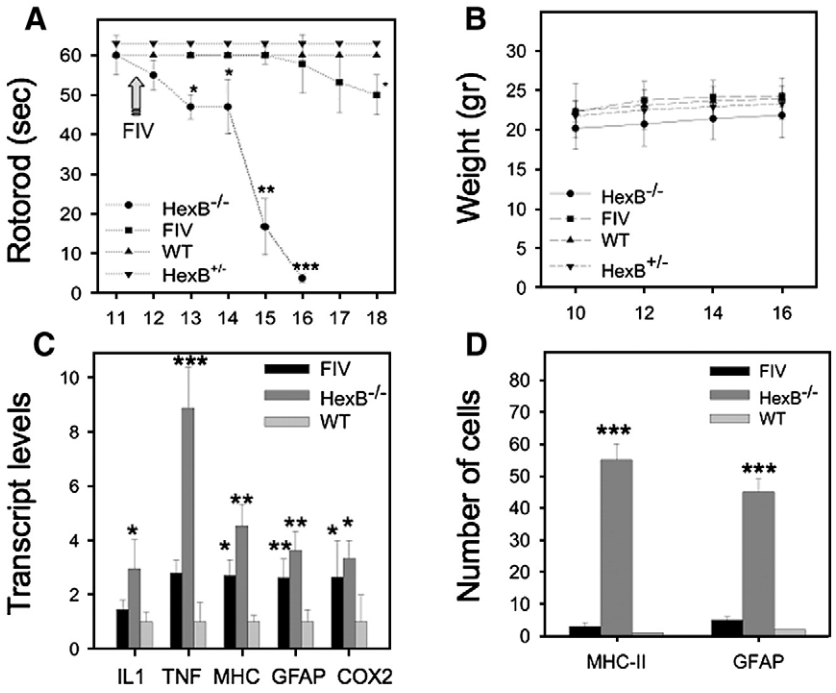

Fig. 2. Trigeminal retrograde FIV(HEX) transfer in HexB$^{-/-}$ adult mice attenuates the disease phenotype. Animal behavior and general health was evaluated longitudinally by rotarod and weight. (A) HexB$^{-/-}$ knockout mice were characterized by motor decline that begun at 13 weeks of age and deteriorated by 18 weeks of age, when the mice had to be euthanized per institutional animal use protocols. Conversely, loss of motor function was attenuated in FIV(HEX)-treated HexB$^{-/-}$ mice (FIV), which only began displaying signs of significant decline at 18 weeks of age. Heterozygous HexB$^{+/-}$ as well as wild type (WT) littermates were included as controls. (B) Weight gain was normal for all groups of mice evaluated. (C) Brain inflammation, as assessed by qRT-PCR for key inflammatory genes, demonstrated a significant reduction in IL-1β and TNFα levels of expression in the FIV(HEX)-treated HexB$^{-/-}$ mice when compared to wild type controls. (D) The number of reactive microglia and astrocytes, assessed by immunohistochemistry, was employed as an additional measure of brain inflammation. We observed that FIV(HEX)-treated HexB$^{-/-}$ mice displayed a significant reduction in the number of MHC-II^{+} and GFAP^{+} positive cells in their brains compared to HexB$^{-/-}$ knockout mice. *** = $p<0.001$; ** = $p<0.01$; * = $p<0.05$.

reactive microglia and astrocytes, as evaluated by MHC-II and GFAP immunohistochemistry, respectively. We also observed that FIV(HEX) treatment administered to adult HexB$^{-/-}$ mice resulted in significant reduction of the number of MHC-II^{+} and GFAP^{+} cells in the brain, to near "normal" levels (Fig. 2D). In addition, CD45+ expression was employed as a marker of activated macrophages/monocytes in the brain parenchyma. As shown previously, HexB$^{-/-}$ mice display large numbers of CD45+ cells in their brain (Kyrkanides et al., 2008). Conversely, FIV(HEX)-treated mice were characterized by the absence of CD45+ cells in their brain (data not shown). Fig. 3 depicts representative examples of GFAP and MHC-II staining in the brain of various animal groups, which were present throughout the brain parenchyma.

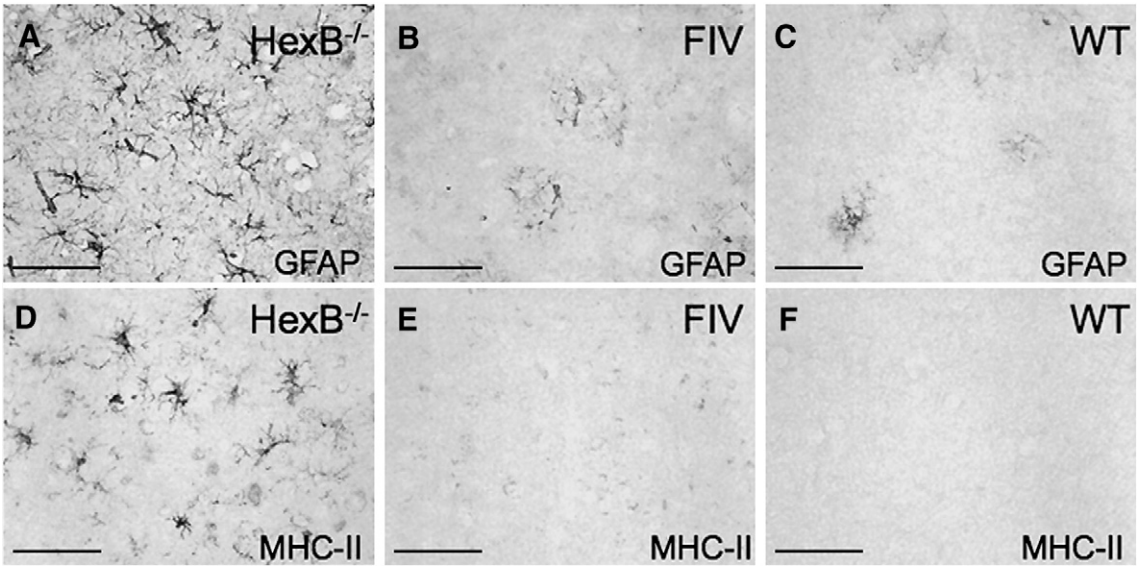

Fig. 3. Trigeminal retrograde FIV(HEX) transfer in HexB$^{-/-}$ adult mice reduces glial cell activation. The images depicted in this figure are from cortical areas. (A) HexB$^{-/-}$ knockout mice were characterized by GFAP upregulation, a marker of reactive astrocytes, throughout the brain parenchyma, including the cortex, as assessed by immunohistochemistry. (B) Conversely, FIV(HEX)-treated HexB$^{-/-}$ mice displayed wild type GFAP immunoreactivity in the brain in comparable sections. (C) Wild type mice are characterized by lack of GFAP^{+} cells in the cortex. (D) MHC-II expression, employed as a marker of microglia activation, was increased in HexB$^{-/-}$ knockout mice. (E) In contrast, MHC-II expression was reduced to wild type levels in the FIV(HEX)-treated HexB$^{-/-}$ mice. (F) Wild type mice do not demonstrate MHC-II immunoreactivity. (40×) Bar = 100 μm.

4. Discussion

Similarly to the human, two genes, HexA and HexB, encode for β-hexosaminidase in the mouse (Sango et al., 1995). Targeted deletion of HexA resulted in the development of a mouse phenotype that showed only a mild degree of the expected pathology and lack of any neurological findings (Yamanaka et al., 1994). In contrast, disruption of the murine HexB locus resulted in a mouse phenotype that closely resembled that of the human disease, showing GM2 storage (Sango et al., 1995). Unlike human Sandhoff patients, $HexB^{-/-}$ mice demonstrated increased levels of GA2, apparently due to a murine sialidase that converts GM2 to GA2 in mouse, which subsequently can be, at least in the absence of HEXA (α/β), catabolized by HEXB (β/β). Conversely, human sialidases cannot metabolize GM2 to GA2 ganglioside.

Our studies demonstrate that FIV(HEX) administration into the TMJ results in retrograde transfer and expression of HEXB in trigeminal sensory neurons. Retrograde transfer along trigeminal nerves has previously been demonstrated in our laboratory employing the reporter gene gfp (Kyrkanides et al., 2004) or other genes of interest (Kyrkanides et al., 2007a). The subsequent amelioration of GM_2 storage in the brain of $HexB^{-/-}$ mice, including in areas (cortex) distal to the region where the primary trigeminal sensory nerves terminate (brain stem) implies the ability of β-hexosaminidase for cross-correction. To this end, the expression HEXB by trigeminal sensory afferents in the brain stem would result in HEXB release in the parenchyma and, via paracrine pathways, the uptake by neighboring cells as well as nerve fibers terminating in that location. For example, descending nerve tracks can potentially enable HEXB cross-correction from the medullary parenchyma to higher brain areas, including the cortex (i.e. cortico-bulbar track). An additional pathway of HEXB cross-correction from the medulla to the brain potentially is through the cerebrospinal fluid (CSF). To this end, as sensory trigeminal afferents terminate close to the dorsal surface of the brain stem, it is possible that HEXB is up-taken by the CSF circulation and subsequently transferred to the brain. To this end, administration of recombinant human iduronidase enzymes into the CSF after intrathecal injection in a dog model of Hurler syndrome proved to be of therapeutic value (Dickson et al., 2007). Moreover, injection of recombinant human sulfamidase into the cerebrospinal fluid via intra-cisterna injection also yielded therapeutic results (Hemsley et al., 2007).

Clinically, $HexB^{-/-}$ knockout mice display, similarly to human patients, near normal phenotype at birth, but quickly develop muscle weakness, rigidity, and motor deterioration typically leading to death at approximately 18 weeks of age (Sango et al., 1995). Therefore, the therapeutic window for the treatment of GM_2 gangliosidosis is temporally narrow and appropriate timing of gene therapy administration is critical for desired levels of therapeutic efficacy. However, a study by Brooks et al. (2002) demonstrated the ability of FIV-based gene therapy to reverse the development of mucopolysaccharidosis type VII in a mouse model of Sly disease following direct intracranial administration in adult animals. Therefore, the attenuation of disease development in our model can be explained by a partial reversal of neuronal GM_2 storage, as $HexB^{-/-}$ mice are characterized by abundant presence of GM_2 ganglioside throughout their brain parenchyma at the age (12 weeks) of intra-articular FIV(HEX) injection (Kyrkanides et al., 2005). The attendant attenuation in brain inflammation, as assessed by the levels of inflammatory gene expression as well as the number of reactive astrocytes and microglia in the brain parenchyma, also indicates restoration of sufficient levels of β-hexosaminidase in the CNS. It is noteworthy that intra-articular FIV(HEX) injections in the TMJ of $HexB^{-/-}$ mice, in contrast with direct intra-cranial or systemic administration of viral gene therapy vectors (Olschowka et al., 2003; Kyrkanides et al., 2007a) did not elicit peripheral blood mononuclear cells infiltration in the brain, as can be seen by the absence of MHC-II+ and CD45+ cells in the brain, nor did it exacerbate the attendant brain inflammation. In contrast, the levels of brain inflammation following treatment were significantly reduced when compared to untreated littermates.

In conclusion, our studies demonstrate the retrograde transport and restoration of β-hexosaminidase in the $HexB^{-/-}$ brain via FIV (HEX) administration in the TMJ. Previous studies evaluating the effectiveness of retrograde transfer of viral gene therapy vectors in a mouse model of infantile neuronal ceroid lipofuscinosis via intraocular injections reported direct therapeutic effects to the eye as well as to distal sites in the brain (Griffey et al., 2005). Therefore, the trigeminal sensory retrograde transport pathway may open new avenues in the delivery of gene therapy vectors for the treatment of lysosomal storage disorders and potentially other neurodegenerative diseases.

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