

Hematopoietic stem cell gene therapy corrects neuronopathic phenotype in murine model of mucopolysaccharidosis type II

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ABSTRACT

Mucopolysaccharidosis type II (MPS II) is a neuropathic lysosomal storage disorder caused by a deficiency of iduronate-2-sulfatase (IDS), which leads to the accumulation of glycosaminoglycans (GAGs). Recently, we demonstrated that biochemical alterations in the brains of MPS II mice are not corrected by bone marrow transplantation (BMT) or enzyme replacement therapy, although BMT has been shown to be effective for other neurodegenerative MPS, such as Hurler syndrome. In this study, we demonstrated that lentiviral isogeneic hematopoietic stem cell (HSC) gene therapy corrected neuronal manifestations by ameliorating lysosomal storage and autophagic dysfunction in the brains of MPS II mice. IDS-transduced HSCs increased enzyme activity both in various visceral organs and the central nervous system (CNS). Decreased levels of GAGs were observed in many organs, including cerebrums, after transplantation of IDS-transduced HSCs. In addition, lentiviral HSC gene therapy normalized the secondary accumulation of autophagic substrates, such as p62 and ubiquitin-protein conjugates, in cerebrums. Furthermore, in contrast to naïve MPS II mice, there was no deterioration of neuronal function observed in transplant recipients. These results indicated that lentiviral HSC gene therapy is a promising approach for the treatment of CNS lesions in MPS II.

1. INTRODUCTION

Mucopolysaccharidosis (MPS) type II, also known as Hunter disease, is the most prevalent MPS in Asian countries. It is an X-linked recessive disorder characterized by a deficiency of lysosomal iduronate-2-sulfatase (IDS), which leads to progressive accumulation of glycosaminoglycans (GAGs), such as heparan sulfate and dermatan sulfate. Intralysosomal accumulation of GAGs induces multiple clinical features including skeletal deformities, sclerema, atherosclerosis, upper airway narrowing, hepatosplenomegaly, cardiac valvular disorder, hypoacusis, and central nervous system (CNS) involvement.

Enzyme replacement therapy (ERT) and hematopoietic stem cell transplantation (HSCT) are currently available to treat patients with MPS II. ERT has been shown to improve some clinical symptoms, such as hepatosplenomegaly and walking capacity^{1,2,3} but not CNS lesions, because enzymes do not cross the blood-brain barrier (BBB)⁴. Additionally, repeated administration of ERT is costly and puts further stress on patients with MPS II. HSCT was the only definitive treatment for MPS II before the development of ERT, and has been shown to correct visceral and central nervous system manifestations of several lysosomal storage diseases including MPS I, globoid-cell leukodystrophy, metachromatic leukodystrophy, and Gaucher disease⁵. Because engrafted donor cells differentiate into both hematopoietic cells and microglial cells that secrete a therapeutic enzyme

allowing for cross-correction of neuronal cells in recipients⁶, HSCT seems to be an effective treatment of neuropathic lysosomal storage disorders. However, early studies reported that there were no beneficial effects of HSCT in CNS disease in patients with MPS II^{7,8,9}. In addition, our previous study demonstrated that bone marrow transplantation (BMT) does not correct biochemical alterations in the brains of MPS II mice, such as the accumulation of GAGs and deficiency of IDS activity¹⁰.

Viral vectors are powerful tools for the transduction of therapeutic genes into various cells. In recent years, *ex vivo* HSC gene therapy with lentiviral vectors has been used as a clinical option for several genetic diseases including adrenoleukodystrophy, metachromatic leukodystrophy, Wiskott-Aldrich syndrome, and β -thalassemia^{11,12,13,14}. Lentiviral *ex vivo* gene therapy has also been shown to be effective for correcting certain aspects of neuronal defects in mouse models of MPS I and MPS IIIA^{15,16}. It appears, therefore, that enhanced secretion of IDS from HSCs is an attractive approach to ameliorate CNS lesions in MPS II. However, the effects of lentivirally-transduced HSCT on CNS disease in MPS II have not been clarified. In this study, we demonstrated that lentiviral isogeneic HSC gene therapy corrected both the biochemical abnormalities in the brain and the deterioration of neurological function in the MPS II mouse model.

2. MATERIALS and METHODS

2.1. Animals

Congenic C57BL/6 female mice¹⁷, which are heterozygous for the IDS gene on the X-linked allele (IDS^{+/-}), were kindly provided by Dr. Joseph Muenzer (University of North Carolina, Chapel Hill, NC). Female heterozygous mice (IDS^{+/-}) were mated with male wild-type mice to generate IDS knockout (KO) male mice (MPS II mice). Their genotypes were determined by PCR¹⁰. The Animal Care and Ethics Committees of the Jikei University School of Medicine approved the animal husbandry and other procedures used in the animal experiments.

2.2. Lentiviral vector construction and production

Codon-optimized human IDS gene (Genscript, Piscataway, NJ) and eGFP gene were inserted into the multiple cloning site of the second generation lentiviral vector pSMPUR-MCU3-MCS¹⁸, which was kindly provided by Dr. Donald B. Kohn (UCLA). Large-scale production of lentiviral vectors was achieved according to a procedure previously described¹⁸. Lentiviral titers were determined by enzyme-linked immunosorbent assay of viral p24 proteins¹⁹.

2.3. Transduction and transplantation of lineage negative bone marrow cells

Whole bone marrow cells were harvested from tibias and femurs of donor MPS II mouse (869 weeks old) and separated into lineage positive and lineage negative populations using the Lineage Cell Depletion Kit (Miltenyi Biotec, Auburn, CA). Lineage-negative cells were transduced at multiplicity of infection 50 with lentiviral vectors carrying IDS or eGFP gene for 16 h on a RetroNectin-coated 6-well plate at a density of 2×10^6 cells /mL in DMEM (Wako Pure Chemicals, Tokyo, Japan) with 10% fetal calf serum, 100 ng/mL rmFlt-3L, 100 ng/mL rmSCF (R&D Systems, Minneapolis, MN), and 8 g/mL polybrene (Sigma-Aldrich, St Louis, MO), as previously described²⁰. The infected cells (2×10^6) were washed with PBS (Wako Pure Chemicals) and injected intravenously into IDS-KO recipient mice (n=14) after lethal irradiation (9 Gy, Hitach-MBR-1520-R irradiator), according to a procedure previously described¹⁰. Blood samples were collected every 4 weeks and mice were perfused and sacrificed 24 weeks after transplantation. Only well perfused tissues were used in all experiments.

2.4. Antibodies

Anti-LC3 antibody was obtained from Novus Biologicals (Littleton, CO). Anti-ubiquitin monoclonal antibody was purchased from Medical and Biological Laboratories (Nagoya, Japan). Anti-ubiquitin polyclonal antibody was obtained from Dako (Glostrup, Denmark). Anti-p62 antibody

and anti-GFP antibody were purchased from Abcam (Cambridge, MA). Anti-actin antibody was obtained from Sigma-Aldrich. Anti-SCMAS (subunit C of mitochondrial ATP synthase) antibody was gifted by Dr. Junji Ezaki (Juntendo University School of Medicine, Japan). Anti-Iba1 antibody was obtained from Wako Pure Chemicals.

2.5. Quantitative analysis of proteins and IDS activity

Protein concentrations were estimated using the BCA Protein Assay Kit (Thermo Fisher Scientific, IL) standardized with bovine serum albumin. IDS activity was assayed using artificial substrate 4-methylumbelliferyl- α -iduronide-2-sulfate, according to methods previously described²¹.

2.6. Total GAGs assay and pathological iduronic acid assay

Total GAGs assays were performed by using Wieslab sGAG quantitative Alcian blue-binding assay kit (Euro-Diagnostica, Malmö, Sweden), as previously described²¹. The pathological iduronic acid (PIA) assay was performed according to the procedure described by Shimada, et al.²². Briefly, GAGs in cerebral homogenates were isolated by an Anion exchange column and digested with recombinant iduronidase and recombinant IDS (gifted by Genzyme Japan).

Disease-specific iduronic acid from the non-reducing terminal of heparan sulfate and dermatan sulfate was derivatized with ethyl 4-amino benzoate (ABEE) by reductive amination, and measured against the standard curves of the authentic compound using high-performance liquid chromatography (HPLC).

2.7. SDS–PAGE and western blot analysis

Cerebral tissues were homogenized in 9 volumes of 50 mM Tris-HCl pH 7.5 (Tris buffer) containing 1% triton X-100 and Protein Inhibitor Cocktail (PIC) (Roche Diagnostics, Indianapolis, IN), followed by sonication and centrifugation at $18,000 \times g$ for 1 h at 4 °C. Residual pellets were lysed with 7 volumes of Tris buffer containing 2% SDS and PIC, followed by sonication and centrifugation, as previously described. The supernatants were identified as the detergent-insoluble fractions. Alternatively, whole tissue extracts from cerebrums were obtained according to a procedure previously described^{23,24}. Samples containing equal amounts of proteins were resolved by SDS-PAGE on 4–20% acrylamide gradient gels and transferred onto nitrocellulose membranes. The membranes were dipped in blocking buffer (Tris buffer containing 150 mM NaCl, 0.1% gelatin, 0.1% casein, and 0.05% Tween 20) and incubated with each primary antibody. After a brief wash,

membranes were immunostained with peroxidase-labeled secondary antibody (Nichirei Corp, Tokyo, Japan) and detected by Immunostar LD (Wako Pure Chemicals).

2.8. Flow cytometry

We used Abs specific for CD11b-PE (clone M1/70), Ly-6G-PE (clone RB6-8C5), CD45R-PE (clone RA3-6B2), and CD3e-PE (clone 145-2C11) (eBioscience, San Diego, CA). Peripheral white blood cells (WBCs) were obtained from recipient mice transplanted with eGFP-overexpressing HSCs. Cells were incubated with Fc blocker (CD16/32; e Bioscience) and stained to obtain the expression of surface markers²⁵. Data were obtained from a MACSQuant Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany) and analyzed using MACSQuantify Software (Miltenyi Biotec).

2.9. Immunohistochemistry

Preparation of brain sections and immunostaining were performed according to a procedure previously described^{21,26}. Sliced sections were incubated with the primary antibodies and visualized by Alexa 555-conjugated anti-rabbit IgG, Alexa 488-conjugated streptavidin (Invitrogen,

Carlsbad, CA), and biotinylated anti-chicken IgY (H+L) (GeneTex, Irvine, CA) secondary antibodies.

2.10. *Quantitative RT-PCR*

Vector copy number in livers and cerebrums were determined by analysis of lentiviral packaging sequences in genomic DNA using quantitative real time PCR, as previously described¹⁹.

2.11. *Y-maze test*

The behaviors of wild type, MPS II, and transplanted MPS II mice (9 and 33 weeks old) were assessed by Y-maze testing as previously described²¹.

2.12. *Statistical analysis*

Statistical analysis was performed using JMP statistical software ver10 (SAS Institute Inc., Cary, NC). The Student's t-test was used in this study. $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1 *Lentiviral ex vivo HSC gene therapy increases IDS activities in MPSII tissues*

To investigate the effect of lentiviral gene delivery to HSCs, we transfected lineage negative cells derived from IDS-deficient bone marrow cells with a lentiviral vector encoding IDS and analyzed the activities of the enzyme. Lentiviral gene transduction drastically increased IDS activity in lineage-negative cells derived from MPS II mice (non-transduced:45.7 nmol/4hr/mg protein vs transduced:7418.9 nmol/4hr/mg protein).

Next, we transplanted lentiviral IDS-transduced MPS II HSCs into lethally irradiated recipient IDS-KO mice and analyzed the activities of the enzyme in samples from several tissues including liver, heart, cerebrum, and serum (N=4~8. respectively). From 2 weeks after transplantation, the levels of IDS activity in serum were increased from 0 nmol/4 h/mg protein to 398.6 nmol/4 h/mg protein and supernormal levels (approximately 15-fold higher than wild type) were sustained throughout the 24 weeks of treatment (Fig. 1A). Supernormal IDS activity was also observed in liver (approximately 11-fold higher than wild type) and heart (approximately 3-fold higher than wild type) derived from recipient MPS II mice (Fig. 1B). Furthermore, transplantation of IDS-overexpressing HSC to MPS II mice significantly increased the activities of the enzyme in

cerebral tissues (approximately 2.9% of wild-type activity) (Fig. 1B). These results indicated that lentiviral *ex vivo* HSC gene therapy increased IDS activity both in peripheral tissues and the CNS in MPS II mice.

3.2 Lentiviral ex vivo HSC gene therapy corrected the accumulation of GAGs in MPSII tissues

To evaluate the therapeutic outcome of IDS-overexpressing isogeneic HSCT, we analyzed the amounts of GAGs in livers, hearts, and cerebrums of recipient MPS II mice (33 weeks old, n=5~8, respectively) by Alcian blue staining. The levels of total GAGs in liver and heart tissue of MPS II mice were normalized by treatment with lentiviral HSC gene therapy (Fig. 2A). In contrast, while the levels of total GAGs in cerebrums were slightly increased in MPS II mice compared to wild type, no significant difference was observed between untreated and transplanted mice (Fig. 2A). We previously demonstrated that Alcian blue staining is not sensitive or specific enough to precisely evaluate the accumulated levels of GAGs in MPS II mouse cerebrums; however, this can be achieved using the pathologic GAGs assay^{10, 27}. In this study, we analyzed PIA derived from the non-reducing end of disease-specific GAGs in cerebrums of MPS II mice (n=5, respectively) because the PIA assay could quantify the amount of disease-specific GAGs as accurately as the pathological GAGs assay²². The PIA assay showed a drastic increase in the levels of PIA in the cerebrums of MPS II

mice and a significant reduction in diseased mice transplanted with lentivirally-transduced HSC (Fig. 2B). These results indicated that lentiviral *ex vivo* HSC gene therapy reduced the accumulation of GAGs both in peripheral tissues and the CNS in MPS II mice.

3.3 *Lentiviral ex vivo HSC gene therapy rescued autophagic dysfunction in MPSII cerebrums*

Defective autophagy is a prominent feature of lysosomal storage disorders²⁸. In MPS, lysosomal enzyme deficiency has been shown to induce retardation of autophagic flux in the brain of the mouse model of MPS IIIA²⁹, which results in heparan sulfate accumulation, similar to the effects in MPS II. To investigate whether impaired autophagy can be observed in the brains of MPS II mice, we analyzed the levels of autophagic marker LC3 in cerebrums using Western blot analysis (n=3, respectively). The levels of LC3-II, a major component of autophagosomes, were higher in MPS II mice than in the wild type (Fig. 3A). We then analyzed autophagy substrates such as p62, detergent-insoluble ubiquitin-protein conjugates, and SCMAS. The levels of both p62 and Triton X-100-insoluble ubiquitin-protein conjugates were increased in MPS II mice cerebrums compared with the wild-type (Fig. 3A and B). Moreover, immunohistochemical analysis revealed neuronal accumulation of ubiquitin-protein conjugates and SCMAS in cerebral tissues from MPS II mice (Fig. 3C), indicating that autophagic flux is impaired in the MPS II mouse brain.

We subsequently investigated the effects of IDS-overexpressing isogeneic HSCT on autophagic dysfunction in the brains of MPS II mice. The levels of LC3-II, p62, and detergent-insoluble ubiquitinated proteins in MPS II mouse were reduced by treatment with lentiviral HSC gene therapy (Fig. 3A and B). Furthermore, a positive effect of genetically-modified HSCT on neuronal accumulation of ubiquitin-protein conjugates and SCMAS was observed in MPS II mice cerebrums (Fig. 3C). These results indicated that lentiviral *ex vivo* HSC gene therapy rescued autophagic flux in the MPS II mouse cerebrum.

3.4 Lentiviral ex vivo HSC gene therapy prevented neurofunctional deterioration in MPS II mice

MPS II mice were analyzed by the Y-maze test, which evaluates short-term memory performance, to investigate the effect of IDS-overexpressed HSCT on behavioral abnormalities (n=3~5, respectively)^{21,30,31}. Although spontaneous alternation behavior did not differ between MPS II mice and wild-type mice at 9 weeks old, rates were impaired in a time-dependent manner in MPS II mice (Fig. 4). On the other hand, transplantation of IDS-overexpressed HSCs inhibited the deterioration of alternation behavior in MPS II mice (Fig. 4). These results indicated that lentiviral *ex vivo* HSC gene therapy prevented neurofunctional impairments in MPS II mice.

3.5 Gene modified HSCs differentiated into hemocyte fractions in peripheral blood

We analyzed GFP expression in the peripheral blood of recipient mice 12⁶14 weeks after transplantation to confirm whether the transplanted gene-modified HSCs differentiated into hemocyte fractions (n=4). We detected GFP expression in 29.5% of total peripheral WBCs, 31.6% of monocytes (CD11b positive), 52.2% of granulocytes (Ly-6G positive), 20.4% of B cells (GD45R), and 27.6% of T cells (CD3e positive). These results indicated that the transplanted gene-modified HSCs differentiated into cell lineages.

3.6 Some gene modified HSCs translocate to the brain and differentiate into microglial cells

To investigate the therapeutic mechanisms of IDS-overexpressing HSCT in the MPS II mouse brain, we analyzed the lentiviral copy number in genomic DNA from cerebral tissues of transplanted mice using quantitative RT-PCR (n=5, respectively). Whereas 0.045 copies per cell was observed in the livers, the average copy number was 0.0063 copies per cell in the cerebrums of MPS II mice treated with IDS-transduced HSCs, which indicated invasion of HSCs to the cerebrums (Fig. 5A).

Next, to determine whether transplanted HSCs differentiate into microglial cells, we transplanted HSCs overexpressing eGFP into MPS II mice, and analyzed the localization of eGFP in

cerebral tissues using fluorescent immunohistochemistry. eGFP fluorescence was observed in cerebrums of recipient mice, and was colocalized with some microglial marker Iba1-positive cells (Fig. 5B). These results strongly suggest that lentiviral *ex vivo* HSC gene therapy imparts a therapeutic effect in CNS disease in MPS II mice through the differentiation of HSCs into microglial cells, which allows for enzymatic cross-correction.

4.DISCUSSION

We showed that lentiviral *ex vivo* HSC gene therapy improved the biochemical abnormalities in diseased tissues in MPS II mice, including within the cerebrum (Fig. 1). In addition, we found that gene-corrected HSCs rescued autophagic flux retardation and neurofunctional impairments in the CNS of MPS II mice. To our knowledge, this is first report to show that *ex vivo* gene therapy with lentiviral vectors can correct CNS disease in MPS II.

HSCT was shown to ameliorate disease symptoms of MPS I in the 1980s, and is now a standard treatment for the disorder³². In particular, HSCT alleviates several manifestations including neurological deterioration in patients with MPS I when performed at an early age (< 2 years old)³³. HSCT became a treatment option for MPS II patients, since MPS II is similar to MPS I in terms of the accumulation of heparan sulfate and neurodegenerative disease, and a retrospective study of

Japanese MPS IIB patients showed efficacy in the treatment of CNS and valvular lesions³⁴. However some studies have reported that transplantation of HSCs is not effective for CNS disease in patients with MPS II^{7,8,9} and this notion is supported by our recent study, which showed that BMT of wild-type donor cells cannot correct biochemical abnormalities, including GAG accumulation and enzyme activity defects, in the brains of MPS II mice¹⁰. The efficacy of BMT in MPS II patients remains controversial. On the other hand, this study demonstrated that lentiviral isogenic HSC gene therapy improved biochemical and neuropathological alterations in the cerebrums of MPS II mice (Fig. 1B, 2, 3), suggesting that the levels of IDS secreted by naïve HSCs are not sufficient to rescue CNS lesions in MPS II. Indeed, IDS-overexpressing HSCT leads to supraphysiological enzyme activity in the sera of MPS II mice (Fig.1A), whereas transplantation of allogeneic normal bone marrow cells achieves only 20% of the enzymatic activity of wild-type enzymes¹⁰. It appears, therefore, that *ex vivo* gene therapy with lentiviral vectors can overcome the limits of HSCT in MPS II.

Our study showed that lentiviral *ex vivo* HSC gene therapy increased the levels of IDS activity in the cerebrums as well as in liver and heart tissues of MPS II mice (Fig. 1B). Some transplanted HSCs pass through the BBB and differentiate into microglial cells, hence HSCT has been shown to cure several neuropathic lysosomal storage diseases including metachromatic

leukodystrophy (MLD)⁵. Indeed, we found the presence of vector sequences in genomic DNA from cerebral tissues of MPS II mice treated with IDS-corrected HSCT (Fig. 4A). Additionally, we detected the colocalization of eGFP with microglial marker Iba1 in the cerebrums of MPS II mice transplanted with eGFP-overexpressing HSCs (Fig. 4B). Therefore, it seems that the increased levels of IDS activity in the cerebrums of MPS II recipients might be attributed to cross-correction via HSC-derived microglial cells. Furthermore, another contributing factor to the increase in enzyme levels in the CNS could result from enforced passage through the BBB due to high levels of enzymes in the serum. Our study showed that despite donor chimerism in total peripheral blood was approximately 30%, the levels of IDS activity in serum of MPS II after lentiviral HSCs gene therapy were 15-fold higher than that of WT, indicating the levels of enzyme produced per IDS-overexpressed cell might be approximately 50-fold higher than that of normal blood cells. A previous study showed that 5-fold higher enzyme levels in the sera of wild-type mice, achieved by AAV2/5 *in vivo* gene therapy, could pass through the BBB in MPS II mice³⁵ and systemic administration of high-dose recombinant IDS could transiently raise enzyme levels in the brain³⁶. Our treated mice showed more than 15-fold greater enzyme activity continuously in serum, which may have contributed to the increased enzyme levels in the CNS.

Our results showed that transplantation of IDS-overexpressing HSCs improved enzyme

activity in the cerebrums of MPS II mice to approximately 2.9% of the activity found in the wild type, though clearly this is not a dramatic increase (Fig. 1B). Consistent with the results of the enzyme activity assays, we confirmed that very small numbers of lentiviral vector copy and eGFP-positive microglial cells were present in the cerebrums of MPS II recipient mice (Fig. 5). These observations suggested that only a few transplanted-HSCs were recruited to the CNS in our MPS II mice. In wild-type mice, HSCT is reported to replace less than 5% of cerebral microglial cells with cells of HSC origins³⁷. Moreover, the recruitment of HSCs to the brain has been shown to be promoted by neuronal injury and neurodegeneration accompanied by the production of cytokines and chemokines^{6,37,38,39}. It seems that the low recruitment of HSCs into the MPS II brain may be due to insufficient neurodegeneration for triggering the replacement of microglial cells in recipient mice. Our results support this notion because we found no obvious differences in neuronal function between wild-type and pretransplanted MPS II mice (Fig. 4). In this study, we detected the GFP expression in 31.6% of CD11b-positive cells from MPS II mice after transplantation of gene-corrected HSCs. Biffi et al. reported the expression of GFP in approximately 80% of CD11b-positive cells and in approximately 25% of microglial cells derived from MLD mice transplanted with lentivirally GFP-transduced HSCs⁴⁰. They also showed that approximately 10% of wild-type enzyme activity was observed in cerebrum of MLD mice which treated with lentiviral

HSCs gene therapy⁴¹. Therefore, transduction efficacy of our vector to HSCs may be another contributing factor to relatively low IDS activity in brains of MPS II mice transplanted with IDS-overexpressing HSCs. Additionally, a previous report showed that lentiviral *ex vivo* gene therapy using CD11b promoter achieved high GFP expression in CD11b positive cells and more than 10% of wild-type N-sulfoglucosamine sulfohydrolase (SGSH) activity in treated MPS IIIA mice brains⁴². This method could contribute to an improvement in our results.

We found that approximately 2.9% of wild-type IDS activity decreased the amounts of PIA by about 65% in cerebrums of MPS II mice, but did not result in complete normalization (Fig. 2). In several lysosomal storage diseases such as GM1 gangliosidosis and MPS IVB, at least 10% of normal enzyme activity is required to clear the accumulated substrates and prevent the onset of disease^{43,44}. Moreover, the above level of sulfamidase activity normalized the accumulation of heparan sulfate in the brains of MPS IIIA mice transplanted with lentiviral gene modified HSCs¹⁶. It appears, therefore, that nearly 10% of normal IDS activity is required to achieve complete clearance of PIA in MPS II, which is similar to other lysosomal storage diseases. On the other hand, our results revealed that a small amount of IDS activity improved the accumulation of autophagy-related proteins such as LC3-II, p62, detergent-insoluble ubiquitin-protein conjugates, and SCMAS in the cerebrums of MPS II mice (Fig. 3). In recent years, excessive lysosomal accumulation of undegraded

substrates has been shown to induce defective lysosome-autophagosome fusion and block autophagic flux in many lysosomal storage diseases, including MPS IIIA and multiple sulfatase deficiency²⁹.

Secondary accumulation of autophagic substrates, rather than the primary accumulation of lysosomal substrates, is regarded as a key determinant of cell death. Consistent with the above model of disease pathogenesis, we found that IDS activity prevented not only secondary accumulation of p62, detergent-insoluble ubiquitin-protein conjugates, and SCMAS but also deterioration in the alternation behavior of MPS II mice treated with HSC gene therapy (Fig. 3 and 4). Hence, it seems that even if IDS does not completely eliminate GAG accumulation, the enzyme may contribute to suppression of disease progression in MPS II through maintaining autophagic flux.

Overexpression of galactocerebrosidase in HSCs is known to be toxic and results in functional impairment and apoptosis⁴⁵. However, IDS-overexpression in HSCs did not cause these problems and we observed reconstitution of each cell lineage. Moreover, we also measured arylsulfatase A and arylsulfatase B in IDS-overexpressing 293A cells, because the activity of other sulfatases can be reduced due to excessive consumption of formylglycine-generating enzyme (FGE)^{46,47}. The activities of these two sulfatases did not reduce this effect (data not shown).

The therapeutic potential and safety of lentiviral HSC gene therapy has been reported for several animal models of lysosomal storage disorders, such as globoid cell leukodystrophy and

Farber disease⁴⁸. In particular, the efficacy of *ex vivo* HSCT as a lentiviral vector for patients with adrenoleukodystrophy and metachromatic leukodystrophy has already been demonstrated in clinical trials^{11,12}, indicating that lentiviral *ex vivo* HSC gene therapy is a realistic therapeutic strategy for lysosomal storage diseases. In this study, we showed that lentiviral gene correction of autologous HSCs from MPS II mice improved the manifestations of neuronal disease including biochemical and functional abnormalities, which are resistant to other therapeutic approaches including allogeneic normal HSCT and ERT. Our results have provided the evidence that lentiviral autologous HSC gene therapy has the potential to become an encouraging option for the treatment of CNS disease in patients with MPS II.

The limitations of this study included gene-toxicity and the confirmation of transduction in stem cells. Gene toxicity is a serious problem due to possibility of inducing malignant tumors via gene therapy in X-linked severe combined immunodeficiency (X-SCID) and chronic granulomatous disease (CGD) patients^{49,50}. Additionally, clonal expansion was observed in β -thalassemia treated by lentiviral *ex vivo* gene therapy⁵¹. We will be conducting further evaluation and analysis of the integration sites in our future studies.

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Contribution: T.W., Y.S., H.K. and T.O. designed and conducted research; T.W., Y.S., T.H., T.F. and H.K. performed research and analyzed the data; T.W. wrote manuscript; and Y.S., H.K., Y.E., H.I. and T.O. discussed data and edited the paper.

6. AUTHORSHIP

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7 AUTHER DISCLOSURE STATEMENT

Conflict of interest: T.O. and Y.E have active research support from Genzyme Japan Co., Ltd. and Shire Japan Co., Ltd. H.I. has active research support from Genzyme Japan Co., Ltd. and Dainippon Sumitomo Co., Ltd. These activities have been fully disclosed and are managed under a

Memorandum of Understanding with the Conflict of Interest Resolution Board of The Jikei

University School of Medicine. T.W., Y.S., K.A., T.H., T.F. and H.K have no potential COI to disclose.

REFERENCES

1. Muenzer J, Wraith JE, Beck M, et al. A phase II/III clinical study of enzyme replacement therapy with idursulfase in mucopolysaccharidosis II (Hunter syndrome). *Genet. Med.* 2006;8(8):465-473.
2. Okuyama T, Tanaka A, Suzuki Y, et al. Japan Elaprase Treatment (JET) study: idursulfase enzyme replacement therapy in adult patients with attenuated Hunter syndrome (Mucopolysaccharidosis II, MPS II). *Mol. Genet. Metab.* 2010;99(1):18-25.
3. Muenzer J, Beck M, Eng CM, et al. Long-term, open-labeled extension study of idursulfase in the treatment of Hunter syndrome. *Genet. Med.* 2011;13(2):95-101.
4. Manara R, Priante E, Grimaldi M, Santoro L, Astarita L. Brain and spine MRI features of Hunter disease : frequency , natural evolution and response to therapy. 2011;763-780.
5. Malatack JJ, Consolini DM, Bayever E. The status of hematopoietic stem cell transplantation in lysosomal storage disease. *Pediatr. Neurol.* 2003;29(5):391-403.
6. Sano R, Tessitore A, Ingrassia A, d'Azzo A. Chemokine-induced recruitment of genetically modified bone marrow cells into the CNS of GM1-gangliosidosis mice corrects neuronal pathology. *Blood.* 2005;106(7):2259-68.
7. Shapiro EG, Lockman L a, Balthazor M, Krivit W. Neuropsychological outcomes of several storage diseases with and without bone marrow transplantation. *J. Inherit. Metab. Dis.* 1995;18(4):413-29.
8. Vellodi a, Young E, Cooper a, et al. Long-term follow-up following bone marrow transplantation for Hunter disease. *J. Inherit. Metab. Dis.* 1999;22(5):638-48.
9. Guffon N, Bertrand Y, Forest I, Fouilhoux A, Froissart R. Bone marrow transplantation in children with Hunter syndrome: outcome after 7 to 17 years. *J. Pediatr.* 2009;154(5):733-67.
10. Akiyama K, Shimada Y, Higuchi T, et al. Enzyme augmentation therapy enhances the therapeutic efficacy of bone marrow transplantation in mucopolysaccharidosis type II mice. *Mol. Genet. Metab.* 2013;

11. Cartier N, Hacein-Bey-Abina S, Bartholomae CC, et al. Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science*. 2009;326(5954):818623.
12. Biffi A, Montini E, Lorioli L, et al. Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy. *Science*. 2013;341(6148):1233158.
13. Aiuti A, Biasco L, Scaramuzza S, et al. Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome. *Science*. 2013;341(6148):1233151.
14. Drakopoulou E, Papanikolaou E, Anagnou NP. The Ongoing Challenge of Hematopoietic Stem Cell-Based Gene Therapy for α -Thalassemia. *Stem Cells Int*. 2011;2011:987980.
15. Visigalli I, Delai S, Politi LS, et al. Gene therapy augments the efficacy of hematopoietic cell transplantation and fully corrects mucopolysaccharidosis type I phenotype in the mouse model. *Blood*. 2010;116(24):513069.
16. Langford-Smith A, Wilkinson FL, Langford-Smith KJ, et al. Hematopoietic stem cell and gene therapy corrects primary neuropathology and behavior in mucopolysaccharidosis IIIA mice. *Mol. Ther*. 2012;20(8):1610621.
17. Garcia a R, Pan J, Lamsa JC, Muenzer J. The characterization of a murine model of mucopolysaccharidosis II (Hunter syndrome). *J. Inherit. Metab. Dis*. 2007;30(6):924634.
18. Kobayashi H, Carbonaro D, Pepper K, et al. Neonatal gene therapy of MPS I mice by intravenous injection of a lentiviral vector. *Mol. Ther*. 2005;11(5):776689.
19. Kimura T, Koya RC, Anselmi L, et al. Lentiviral vectors with CMV or MHCII promoters administered in vivo: immune reactivity versus persistence of expression. *Mol. Ther*. 2007;15(7):139069.
20. Falahati R, Zhang J, Flebbe-Rehwaltd L, et al. Chemoselection of allogeneic HSC after murine neonatal transplantation without myeloablation or post-transplant immunosuppression. *Mol. Ther*. 2012;20(11):218069.
21. Higuchi T, Shimizu H, Fukuda T, et al. Enzyme replacement therapy (ERT) procedure for mucopolysaccharidosis type II (MPS II) by intraventricular administration (IVA) in murine MPS II. *Mol. Genet. Metab*. 2012;107(1-2):12268.

22. Shimada Y, Wakabayashi T, Akiyama K, et al. Development of novel analytical method for measuring disease-specific iduronic acid in mucopolysaccharidosis type II. Submitted to *Mol. Genet. Metab.*
23. Shimada Y, Fukuda T, Aoki K, et al. A protocol for immunoaffinity separation of the accumulated ubiquitin-protein conjugates solubilized with sodium dodecyl sulfate. *Anal. Biochem.* 2008;377(1):77682.
24. Shimada Y, Nishimura E, Hoshina H, et al. Proteasome Inhibitor Bortezomib Enhances the Activity of Multiple Mutant Forms of Lysosomal α -Glucosidase in Pompe Disease. *JIMD Rep.* 2014;
25. Ohashi T, Iizuka S, Shimada Y, et al. Administration of anti-CD3 antibodies modulates the immune response to an infusion of α -glucosidase in mice. *Mol. Ther.* 2012;20(10):1924631.
26. Fukuda T, Shimizu J, Furuhashi H, et al. Overexpression of heat shock proteins in pallido-nigral axonal spheroids of nonhuman aged primates. *Acta Neuropathol.* 2005;110(2):145650.
27. Lawrence R, Brown JR, Al-Mafraji K, et al. Disease-specific nonreducing end carbohydrate biomarkers for mucopolysaccharidoses. *Nat. Chem. Biol.* 2012;8(2):1976204.
28. Lieberman AP, Puertollano R, Raben N, et al. Autophagy in lysosomal storage disorders. *Autophagy.* 2012;8(5):719630.
29. Settembre C, Fraldi A, Jahreiss L, et al. A block of autophagy in lysosomal storage disorders. *Hum. Mol. Genet.* 2008;17(1):119629.
30. Sibille EL, Pavlides C, Fenster RJ, McEwen BS. Impaired hippocampal-dependent learning and functional abnormalities in the hippocampus in mice lacking serotonin 1A receptors. 2000;97(26):14731614736.
31. Washida K, Ihara M, Nishio K, et al. Nonhypotensive dose of telmisartan attenuates cognitive impairment partially due to peroxisome proliferator-activated receptor- γ activation in mice with chronic cerebral hypoperfusion. *Stroke.* 2010;41(8):17986806.
32. Muenzer J, Wraith JE, Clarke L a. Mucopolysaccharidosis I: management and treatment guidelines. *Pediatrics.* 2009;123(1):19629.

33. Staba S, Escolar M, Poe M. Cord-blood transplants from unrelated donors in patients with Hurler's syndrome. ... *Engl. J.* 2004;196061969.
34. Tanaka A, Okuyama T, Suzuki Y, et al. Long-term efficacy of hematopoietic stem cell transplantation on brain involvement in patients with mucopolysaccharidosis type II: a nationwide survey in Japan. *Mol. Genet. Metab.* 2012;107(3):513620.
35. Polito VA, Cosma MP. IDS crossing of the blood-brain barrier corrects CNS defects in MPSII mice. *Am. J. Hum. Genet.* 2009;85(2):2966301.
36. Polito VA, Abbondante S, Polishchuk RS, et al. Correction of CNS defects in the MPSII mouse model via systemic enzyme replacement therapy. *Hum. Mol. Genet.* 2010;19(24):4871685.
37. Priller J, Flügel a, Wehner T, et al. Targeting gene-modified hematopoietic cells to the central nervous system: use of green fluorescent protein uncovers microglial engraftment. *Nat. Med.* 2001;7(12):1356661.
38. Priller J, Prinz M, Heikenwalder M, et al. Early and rapid engraftment of bone marrow-derived microglia in scrapie. *J. Neurosci.* 2006;26(45):11753662.
39. Burrell K, Hill RP, Zadeh G. High-resolution in-vivo analysis of normal brain response to cranial irradiation. *PLoS One.* 2012;7(6):e38366.
40. Biffi A, De Palma M, Quattrini A, et al. Correction of metachromatic leukodystrophy in the mouse model by transplantation of genetically modified hematopoietic stem cells. *J. Clin. Invest.* 2004;113(8):1118629.
41. Biffi A, Capotondo A, Fasano S, et al. Gene therapy of metachromatic leukodystrophy reverses neurological damage and deficits in mice. *J. Clin. Invest.* 2006;116(11):3070682.
42. Sergijenko A, Langford-Smith A, Liao AY, et al. Myeloid/Microglial driven autologous hematopoietic stem cell gene therapy corrects a neuronopathic lysosomal disease. *Mol. Ther.* 2013;21(10):1938649.
43. Suzuki Y, Ogawa S, Sakakibara Y. Chaperone therapy for neuronopathic lysosomal diseases: competitive inhibitors as chemical chaperones for enhancement of mutant enzyme activities. *Perspect. Medicin. Chem.* 2009;3:7619.

44. Conzelmann E, Sandhoff K. Partial enzyme deficiencies: residual activities and the development of neurological disorders. *Dev. Neurosci.* 6(1):58671.
45. Visigalli I, Ungari S, Martino S, et al. The galactocerebrosidase enzyme contributes to the maintenance of a functional hematopoietic stem cell niche. *Blood.* 2010;116(11):1857666.
46. Anson DS, Muller V, Bielicki J, Harper GS, Hopwood JJ. Overexpression of N-acetylgalactosamine-4-sulphatase induces a multiple sulphatase deficiency in mucopolysaccharidosis-type-VI fibroblasts. *Biochem. J.* 1993;294 (Pt 3):657662.
47. Capotondo a, Cesani M, Pepe S, et al. Safety of arylsulfatase A overexpression for gene therapy of metachromatic leukodystrophy. *Hum. Gene Ther.* 2007;18(9):821636.
48. Wagemaker G. Lentiviral hematopoietic stem cell gene therapy in inherited metabolic disorders. *Hum. Gene Ther.* 2014;25(10):86265.
49. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, et al. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science.* 2003;302(5644):41569.
50. Stein S, Ott MG, Schultze-Strasser S, et al. Genomic instability and myelodysplasia with monosomy 7 consequent to EVI1 activation after gene therapy for chronic granulomatous disease. *Nat. Med.* 2010;16(2):1986204.
51. Cavazzana-Calvo M, Payen E, Negre O, et al. Transfusion independence and HMGA2 activation after gene therapy of human α -thalassaemia. *Nature.* 2010;467(7313):318622.

Figure legends

Fig. 1. Lentiviral *ex vivo* HSC gene therapy increases IDS activity in MPS II tissues. (A) Sustained IDS activity in sera from MPS II mice transplanted with gene-corrected HSC. Sera was collected from wild-type (WT), MPS II (NT), and HSC-transplanted MPS II (TX) mice every 2 weeks after treatment. IDS activities were expressed as nmol/4 h/ μ L serum (mean \pm SD, n=4). (B) Increased IDS activities in tissues of MPS II mice transplanted with gene-corrected HSC. Cerebrum, liver, and heart tissues were obtained from WT, NT, and TX mice 24 weeks after treatment. IDS activities in each tissue were assayed and designated as nmol/4 h/mg protein (mean \pm SD, n=568). Statistical analyses of the data were performed by Student's t-tests (* : $p < 0.01$).

Fig. 2. Lentiviral *ex vivo* HSC gene therapy corrects the accumulation of GAGs in MPS II tissues. (A) Total GAGs in several tissues from MPS II mice transplanted with gene-corrected HSC. Total GAGs in cerebrums, livers, and hearts derived from wild-type (WT), MPS II (NT), and HSC-transplanted MPS II (TX) mice were measured by Alcian blue-binding assay. The amounts of total GAGs were expressed as μ g/mg protein (mean \pm SD, n=568). (B) Pathological iduronic acid (PIA) in cerebrums from MPS II mice transplanted with gene-corrected HSC. PIA in cerebral tissues from WT, NT, and TX mice was measured using

the PIA assay as described in the Materials and Methods. The amounts of PIA were designated as pmol/mg protein (mean \pm SD, n= 5). Statistical analysis of data were performed using the Student's t-test (* : p < 0.01).

Fig. 3. Lentiviral *ex vivo* HSC gene therapy rescues the autophagic dysfunction in the MPSII brain.

(A) Autophagy related proteins in brains of MPS II mice transplanted with gene-corrected HSC. Cerebral extracts derived from wild-type (WT), MPS II (NT), and HSC-transplanted MPS II (TX) mice were resolved by SDS-PAGE, blotted, and probed with antibodies against LC3, p62, and β -actin. The level of β -actin was analyzed as the internal control. The band intensities of both LC3 and p62 were quantified by densitometry and LC3-II was normalized to LC3-I (mean \pm SD, n=3) . Densitometric values in the histograms of LC3-II/LC3-I and p62 are expressed as fold-changes relative to the wild type, which was assigned a value of 1. Statistical analysis of data were performed using the Student's t-test (* : p < 0.01, **: p<0.05).

(B) Detergent-insoluble ubiquitin-protein conjugates (Ub) in cerebrums of MPS II mice transplanted with gene-corrected HSC. Triton X-100 insoluble fractions derived from WT, NT, and TX were analyzed by Western blot using anti-ubiquitin antibody and anti- β -actin antibody.

(C) Immunohistochemical analysis of Ub and SCMAS in the cerebrums of MPS II mice transplanted with gene-corrected HSC. Cerebral sections derived from WT, NT, and TX

mice were stained by each antibody as described in the Materials and Methods. Both Ub positive cells and SCMAS positive cells in cerebral cortex were counted at 400x magnification (high power field (HPF)) (n=5) and statistical analysis of data were performed using the Student's t-test (* : $p < 0.01$).

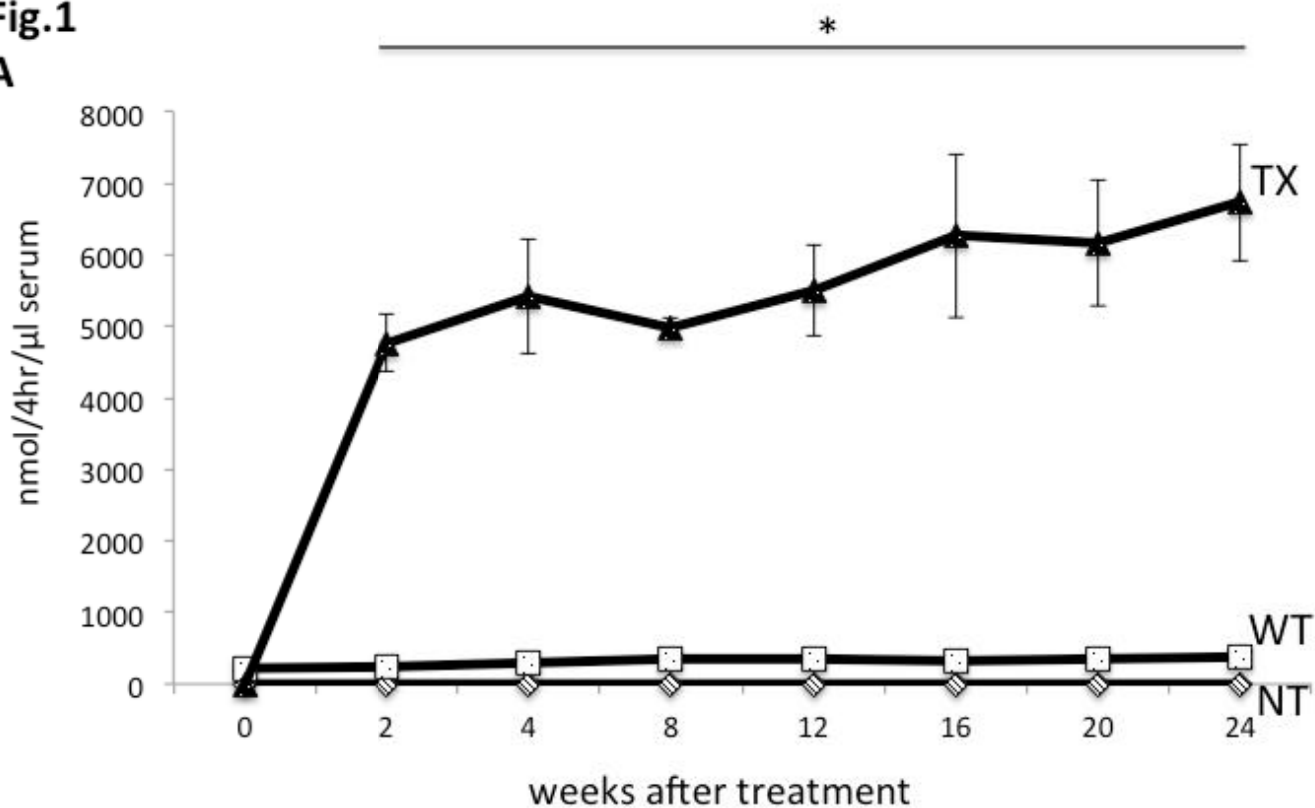
Fig. 4. Lentiviral autologous HSC gene therapy prevents neurofunctional deterioration in MPS II mice. Spontaneous alternation behaviors of wild-type (WT), MPS II (NT), and HSC-transplanted MPS II (TX) mice were analyzed by a Y-maze test before (n=3, respectively) and at 24 weeks after treatment (n=5, respectively). Data are expressed as means \pm SD. Statistical analyses of data were performed by Student's t-test (* : $p = 0.01$, ** : $p < 0.05$).

Fig. 5. Gene-corrected HSCs translocate to the brain and differentiate into microglial cells in MPS II mice. (A) The copy number of lentiviral vectors in MPS II tissues transplanted with gene-corrected HSC. The lentiviral copy numbers in liver and cerebrum were determined by quantitative PCR 24 weeks after transplantation. The limit of detection is 0.0001 copies /cell. (B) Microglial cells derived from transplanted HSCs in MPS II brain. Cerebral sections from MPS II mice transplanted with (TX) or without (NT) lentivirally eGFP-transduced HSCs were stained with antibodies against eGFP and Iba1.

figures

Fig.1

A



B

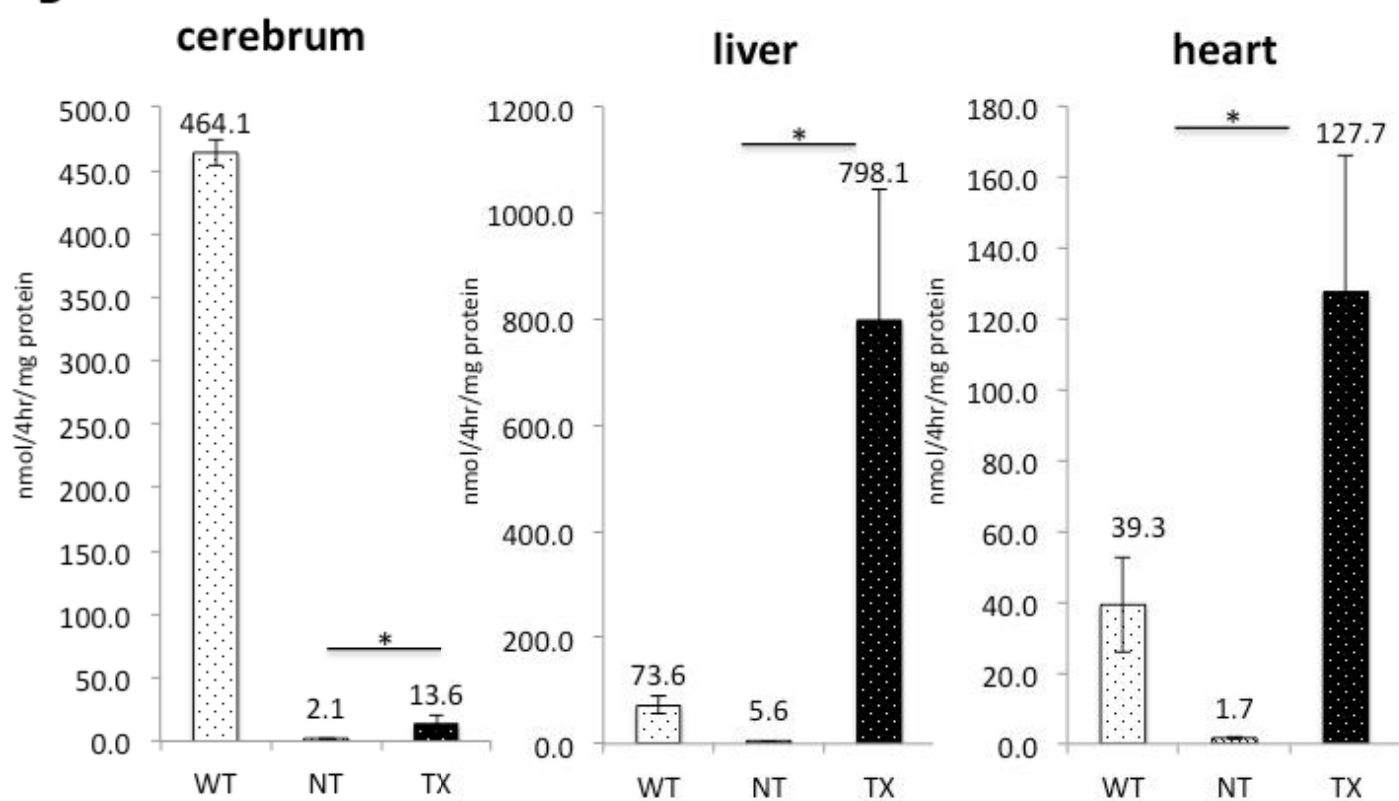
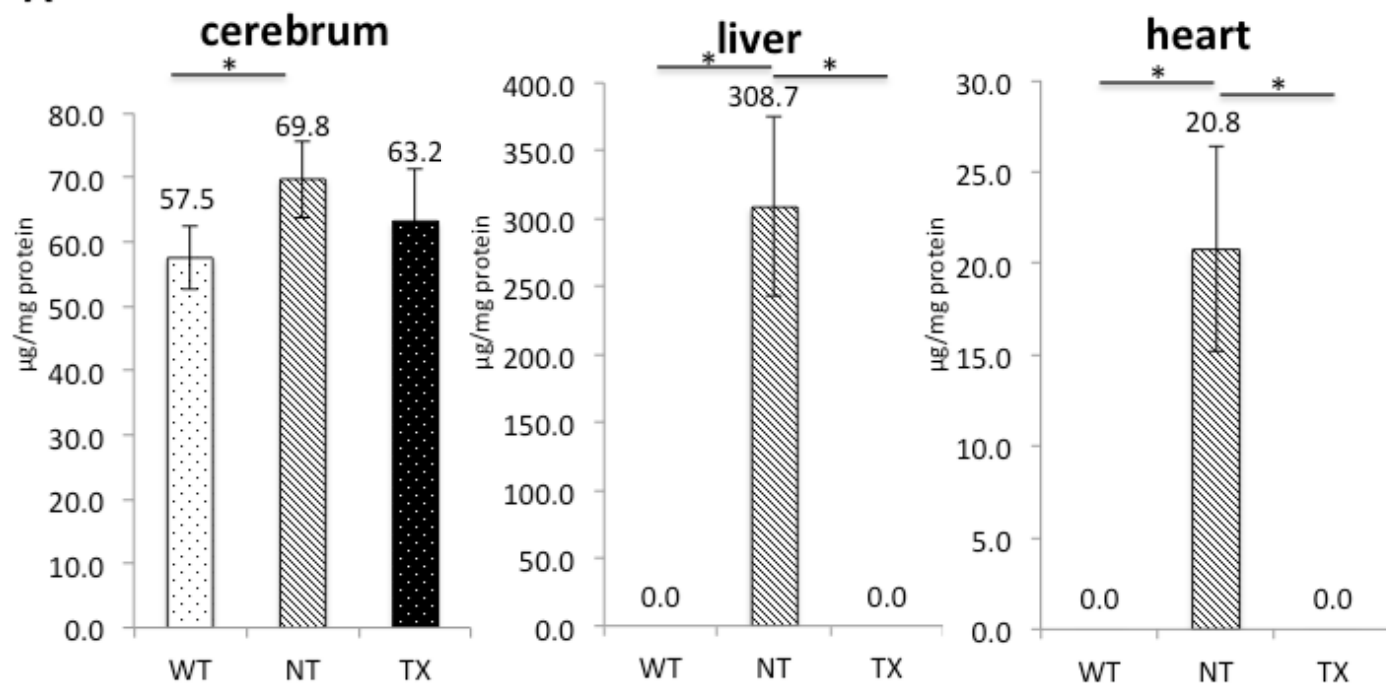


Fig.2

A



B

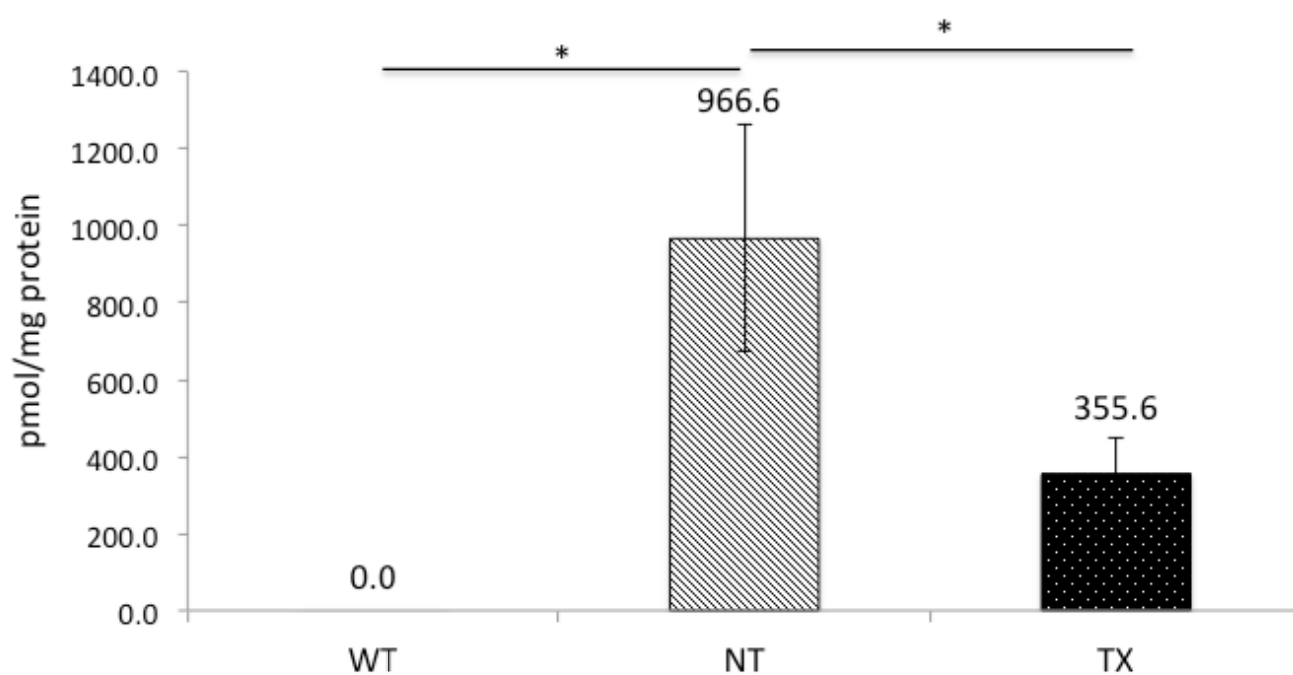


Fig.3

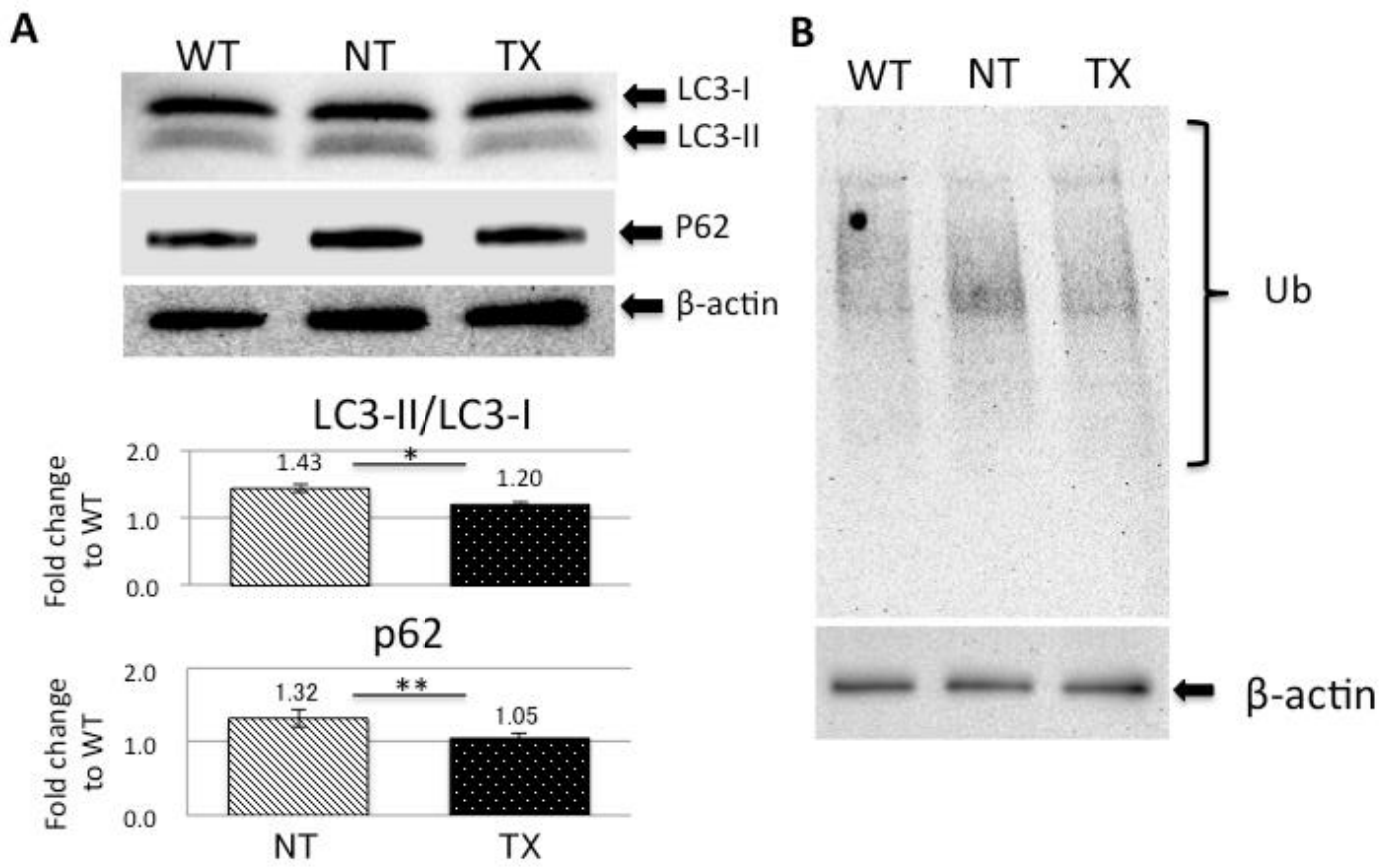
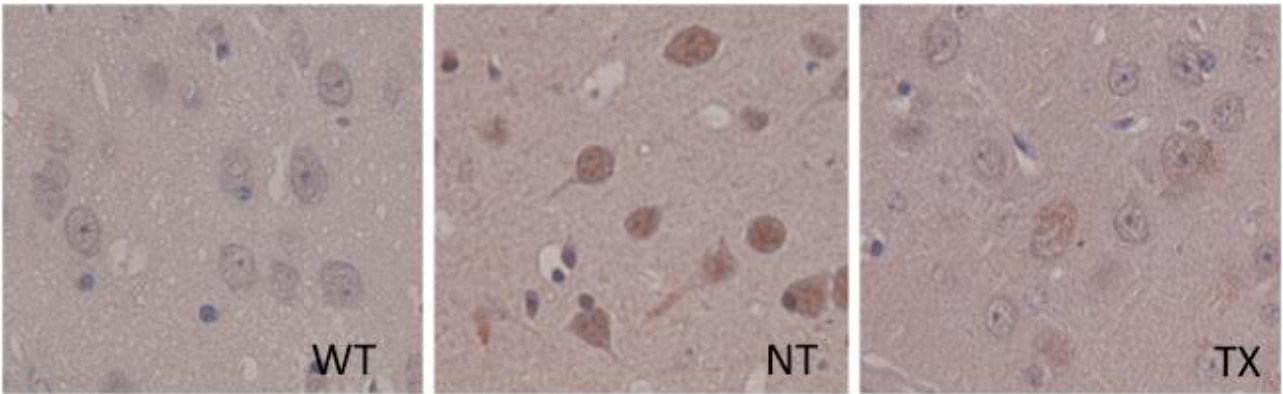


Fig.3

C

Ub



SCMAS

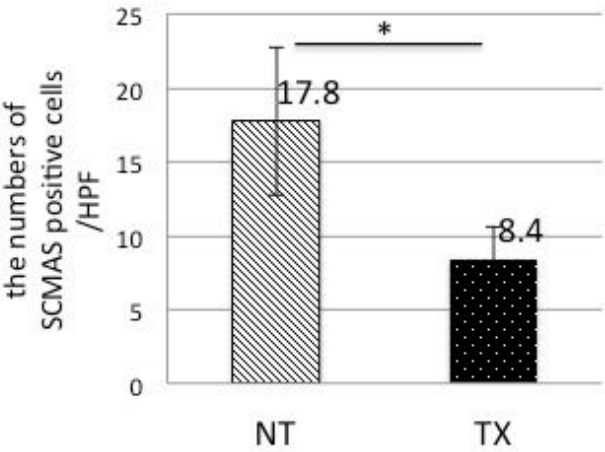
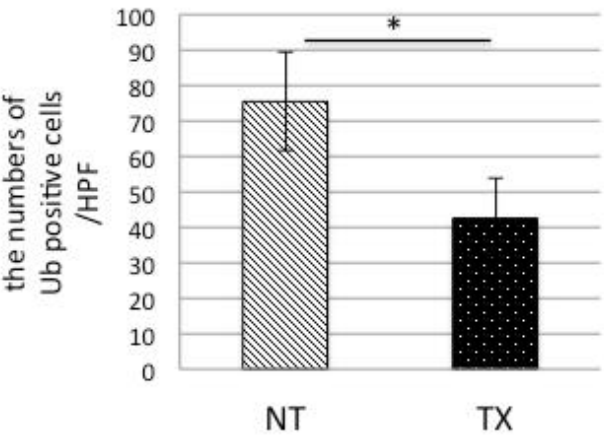
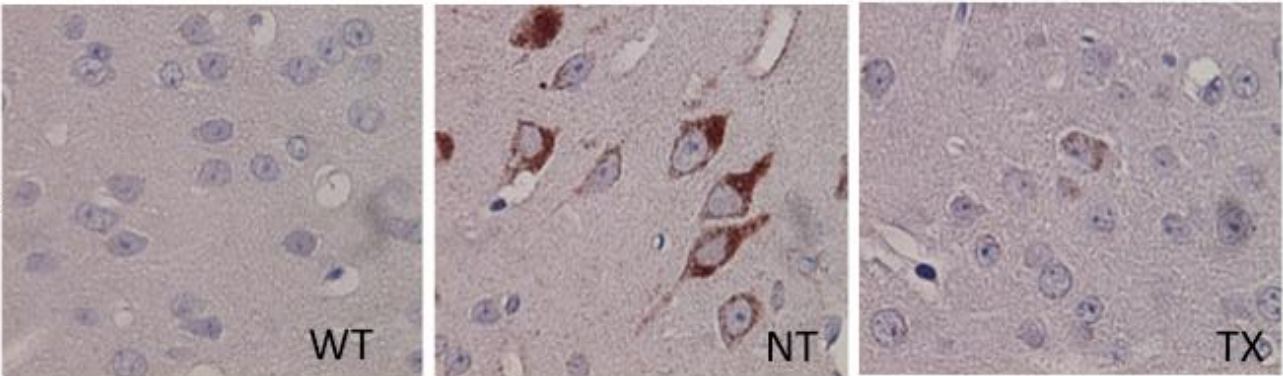


Fig.4

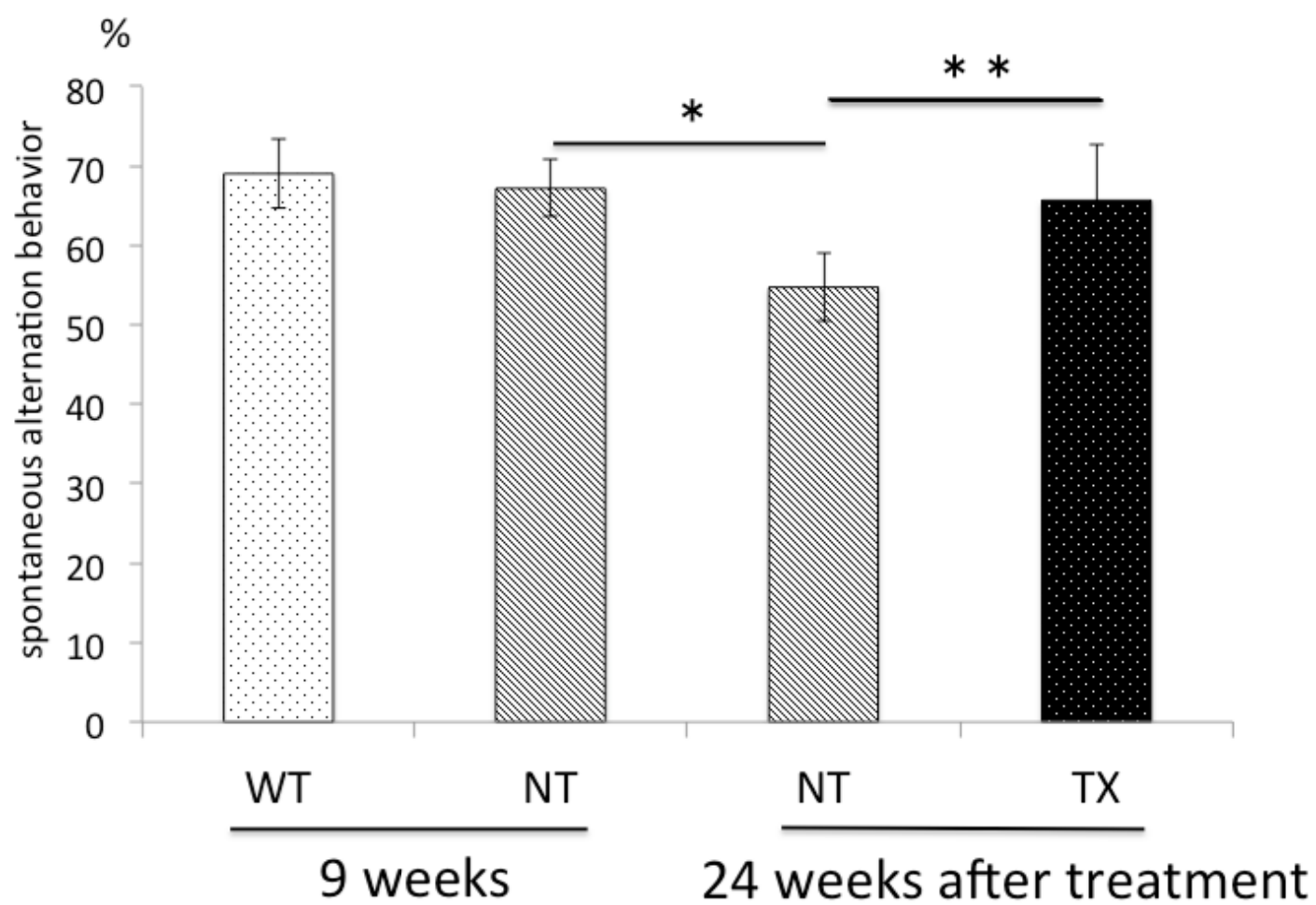
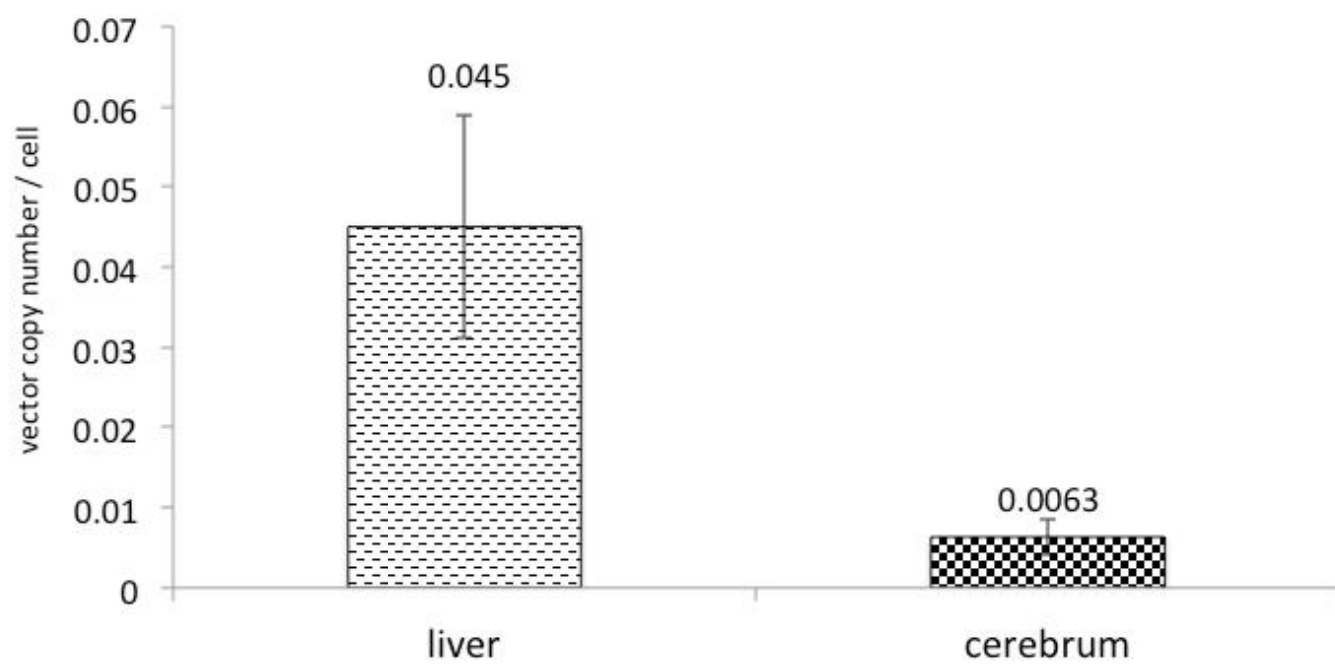


Fig.5

A



B

