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RESEARCH ARTICLE

Widespread and highly persistent gene transfer to the CNS by retrovirus vector *in utero*: implication for gene therapy to Krabbe disease

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Abstract

Background Brain-directed prenatal gene therapy may benefit some lysosomal storage diseases that affect the central nervous system (CNS) before birth. Our previous study showed that intrauterine introduction of recombinant adenoviruses into cerebral ventricles results in efficient gene transfer to the CNS in the mouse. However, transgene expression decreased with time due to the non-integrative property of adenoviral vectors. In this study, in order to obtain permanent gene transduction, we investigated the feasibility of retrovirus-mediated *in utero* gene transduction.

Methods Concentrated retrovirus encoding the LacZ gene was injected into the cerebral ventricles of the embryos of normal and twitcher mice (a murine model of Krabbe disease) at embryonic day 12. The distribution and maintenance of the transgene expression in the recipient brain were analyzed histochemically, biochemically and by the quantitative polymerase chain reaction method pre- and postnatally.

Results Efficient and highly persistent gene transduction to the brain was achieved both in normal and the twitcher mouse. Transduced neurons, astrocytes and oligodendrocytes were distributed throughout the brain. The transduced LacZ gene, its transcript and protein expression in the brain were maintained for 14 months without decrement. In addition, gene transduction to multiple tissues other than the brain was also detected at low levels.

Conclusions This study suggests that brain-directed *in utero* gene transfer using retrovirus vector may be beneficial to the treatment of lysosomal storage diseases with severe brain damage early in life, such as Krabbe disease.

Keywords *in utero*; gene therapy; retrovirus; central nervous system; lysosomal storage disease; twitcher

Introduction

Brain-directed *in utero* gene therapy may benefit lysosomal storage diseases that affect the central nervous system (CNS) before birth. Early treatment is crucial for the treatment of these diseases since the deterioration of the nervous system progresses rapidly after birth and severe brain damage can be irreversible. Gene transfer to produce the deficient enzyme in the brain before birth may prevent the onset and progression of the neuropathology in these diseases. Furthermore, the developing embryonic brain offers unique

advantages for efficient gene delivery to the CNS. It harbors multipotent neural stem cells which are normally inaccessible for gene transduction after birth but can be transduced effectively at early embryonic stage. The subsequent migration of the progeny of the transduced stem cells following normal development would result in wide dispersion of the transgene in the mature CNS.

Recently, we reported brain-targeted *in utero* gene therapy by adenovirus [1]. Recombinant adenoviral vectors administrated into the cerebral ventricles of mouse embryos successfully transduced neural progenitor cells and led to efficient gene transduction to the mature CNS. When a therapeutic gene was introduced into the brain of a mouse model of storage disease by adenovirus, abnormal metabolic storage in the mutant brain was sufficiently prevented. However, the levels of transgene expression and vector genome in the brain decreased with time due to the non-integrative property of the adenovirus vector. Since persistent correction is required for many genetic disorders, it will be more satisfactory to use integrative viral vectors. Retrovirus vector may be the most suitable candidate at present. It has been extensively investigated and widely used in gene therapy studies including human clinical trials. The virus could integrate into host genomes stably, allowing permanent gene transduction to the target cells and their progeny.

Previous studies [2–4] demonstrated that injection of replication-defective retrovirus into sheep and monkey fetus intraperitoneally resulted in the stable transduction of hematopoietic cells and other tissues for several years without detectable pathology. These studies suggested that the direct injection of retroviral vectors *in utero* could be a safe and effective method to achieve permanent gene transduction. Although the technique of gene transfer to embryonic CNS using retrovirus has been widely used in neurobiological studies, such as lineage analysis [5–7] and gene function studies [8,9], the potential of the retrovirus-mediated *in utero* gene transfer to the brain as a therapeutic approach remains unproven. In this study, we injected high-titer retrovirus into mouse embryonic brain intraventricularly and performed detailed analysis of transgene distribution in the mature brain and a long-term study of the presence/expression of transgene by several histochemical and biochemical methods.

The primary motivation of our study on prenatal gene therapy is from an interest in treating globoid cell leukodystrophy (GLD, Krabbe disease), a genetic demyelinating disease caused by genetic defect of β-galactocerebrosidase (GALC) [10]. In this disease, neurological degeneration occurs before birth and progresses rapidly soon after birth causing early death of patients. The cause of demyelination is suggested to be the result of the degeneration or dysfunction of myelin-forming cells (oligodendrocytes and Schwann cells), caused by an accumulation of psychosine, a toxic metabolite of β-galactocerebrosidase [11]. Our study showed that intraventricular injection of retrovirus *in utero* results in sufficient and stable gene transfer into neural cells including oligodendrocytes in normal and twitcher mice (an authentic murine model of Krabbe disease [12]), suggesting the potential usefulness of this approach in treating Krabbe disease and other genetic neurodegenerative disorders.

Materials and methods

Plasmids and virus preparation

A retrovirus vector pLHCX (Clontech, Palo Alto, CA, USA) was modified by replacing the cytomegalovirus (CMV) immediate early promoter with 1.7 kb of CAG hybrid promoter (generated by connecting the CMV enhancer and the modified chicken β-actin promoter [13]). The LacZ gene (excised from plasmid pcDNA3.1/Myc-His/*lacZ*; Invitrogen Corp., Carlsbad, CA, USA) was placed under the CAG promoter to generate a retrovirus vector named pLHCAL where a hygromycin phosphotransferase gene was driven by a long terminal repeat (LTR) and the LacZ gene was driven by the CAG promoter. To produce recombinant retrovirus, the ecotropic packaging cell line ψMP34 [14] was transfected using the calcium phosphate method (Cellphect transfection kit; Amersham Pharmacia, Piscataway, NJ, USA) with pLHCAL. After selection with 0.5 mg/ml of hygromycin B (Wako, Osaka, Japan), a drug-resistant subclone (#44.3) producing the highest titer of LHCAL was isolated and used in this study. Retrovirus was concentrated according the protocol reported by Bowles *et al.* [15]. Briefly, virus-producing cells were grown in 15-cm dishes at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with high glucose (4.5 g/l) supplemented with 5% (v/v) heat-inactivated fetal bovine serum (FBS), and the culture media were changed (18 ml per dish) when cells were confluent. About 10 h after medium change, the virus-containing supernatant (700 ml) was harvested, filtered through 0.22-μm filters (Stericup; Millipore, Bedford, MA, USA) and centrifuged at 6000 *g* at 4 °C overnight. The pellet were resuspended in 1 ml Hanks' balanced salt solution (HBSS) and stored at −85 °C in small aliquots until use.

Viral titer assay and *in vitro* infection

For the determination of viral titers, viral RNA was extracted from viral stocks and their serial dilutions using the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) and eluted in 55 μl of RNase-free water. The reverse-transcription was performed using the SuperScript first-strand synthesis system (Invitrogen) with random hexamer primers in a final volume of 20 μl (8 μl of each viral RNA was used as the template). To determine the copy numbers of viral cDNA, a TaqMan polymerase chain reaction (PCR) for the LacZ gene was performed as described previously [1]. Two microliters of each cDNA was used as the template. As standard controls, 10-fold serial dilutions of known concentration of linearized

plasmid pLHCAL were used. The titers of unconcentrated and concentrated viral supernatant were 9.8×10^8 and 3.9×10^{11} particles per ml, respectively.

For the assessment of the effects of viral titers on the transduction efficiency *in vitro*, NIH/3T3 target cells were grown in 24-well plates (NunC, Roskilde, Denmark) in DMEM supplemented with 10% (v/v) FBS. When approximately 60% confluent, cells were incubated with 0.3 ml medium containing 0.01–10 µl of concentrated LHCAL ($n = 3$) overnight at 37 °C in the presence of 8 µg/ml Polybrene (Sigma, Saint Louis, MO, USA). Forty-eight hours after infection, cells were fixed with 0.25% glutaraldehyde in phosphate-buffered saline (PBS) for 5 min on ice, washed twice with PBS and stained with X-gal solution (1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM $MgCl_2$ in PBS) overnight at 37 °C, and the number of positive cells per mm^2 was counted. The numbers counted from three randomly selected places in each well were averaged to obtain a value per mm^2 for each well.

Animals and *in utero* injection

Breeding pairs of C57BL/6 strain and twitcher heterozygotes (C57BL/6J, twi/+) were purchased from CLEA Japan (Tokyo, Japan) and The Jackson Laboratory (Bar Harbor, ME, USA), respectively, and maintained in our laboratory under standard housing conditions. Genetic status for the twitcher mutation was determined by a PCR method using genomic DNA extracted from the clipped tail [16]. Mice were mated and the day when a vaginal plug was found was designated as embryonic day 0 (E0). *In utero* injection was performed as previously described [17]. Briefly, approximately 2 µl HBSS containing retrovirus (titer see results), Polybrene (concentration see results) and 0.01% Fast Green (Sigma) were injected into the cerebral lateral ventricles of embryos with a heat-pulled glass micropipette at E12.

Tissue preparation and X-gal histochemistry

The mice were anesthetized and fixed with transcardial perfusion of chilled 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) at the time of analysis. Brains were removed and postfixed in the same fixative overnight at 4 °C and cryoprotected in 15% sucrose in 0.1 M PB. Semi-serial coronal sections were made at a thickness of 20–40 µm using a cryostat. Sections were stained overnight at 37 °C with X-gal staining solution as above and counterstained with hematoxylin/eosin (H&E).

To assess the effects of virus and Polybrene on the brain cells of injected mice, brains of wild-type mice with or without viral infection at E12 were harvested at E17. Coronal slices were made and immersion-fixed in 2% paraformaldehyde and 0.25% glutaraldehyde in PBS for 1 h at 4 °C. After washing with PBS, the brain tissues were incubated in X-gal staining solution overnight at 37 °C with 0.02% NP-40 and 0.01% sodium deoxycholate. After a PBS wash, brain slices were dehydrated, cleared and embedded in paraffin. Coronal sections of 3 µm thickness were made, dewaxed and counterstained with H&E (Figures 2d and 2e).

Immunohistochemistry

Tissues were processed as described above. The cryosections (20 µm thick) were incubated with 0.3% Triton X-100 in 0.1 M PBS (PBST) for 1 h at room temperature (RT), then treated with 0.5% H_2O_2 in PBST for 30 min at RT to eliminate endogenous peroxidase activity. Following incubation with 5% normal goat serum (NGS) in PBST for 1 h at RT, the sections were incubated with rabbit polyclonal antibody to β-galactosidase (β-gal; 5 prime → 3 prime Inc., Boulder, CO, USA) diluted 1 : 500 in 3% NGS/PBST overnight at 4 °C. The sections were then incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) diluted 1 : 200 in 3% NGS/PBST for 1h at RT, followed by incubation for 30 min with avidin-biotin complex (ABC) reagent (ABC Elite Kit; Vector Laboratories) at RT. The peroxidase labeling was visualized with 3,3′-diaminobenzidine (DAB) substrate (Vector Laboratories) and sections were dehydrated, cleared and mounted.

For the analysis of proportions of transduced neural cells, the number of each cell type was counted on brain sections stained for β-gal immunohistochemistry. The sections from three 1-month-old recipients were analyzed. The coronal sections corresponding to level 30 in the *'Brain Maps: Structure of the Rat Brain'* by Swanson [18] were used to analyze the cerebral cortex, the hippocampal formation and the diencephalon and level 55 for the brain stem and the cerebellar white matter.

Immunofluorescence was performed as previously described [1,19]. The following primary antibodies were used: rabbit polyclonal anti-phosphorylated histone H3 (Upstate Biotechnology, Lake Placid, NY, USA; 1 : 100 dilution), rabbit polyclonal anti-β-gal (5 prime → 3 prime Inc.; 1 : 100), mouse monoclonal anti-β-gal (Promega, Madison, WI, USA; 1 : 500), mouse monoclonal anti-neuronal nuclei (NeuN; Chemicon, Temecula, CA, USA; 1 : 100), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; Dako, Glostrup, Denmark; 1 : 300), rabbit polyclonal anti-pi-form of glutathione-S-transferase (GST-π; MBL, Nagoya, Japan; 1 : 500) and rabbit polyclonal anti-myelin basic protein (MBP; Dako; 1 : 500). The secondary antibodies used were: FITC-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG (Cappel, Aurora, OH, USA; 1 : 100). The sections were viewed under a fluorescence microscope and photographed using an AquaCosmos CCD camera (Hamamatsu Photonics, Hamamatsu, Japan).

β-Galactosidase enzyme assay

β-Gal activities of the recipients' whole brain were measured using the β-galactosidase enzyme assay kit (Promega). The brains were homogenized in 10 volumes of distilled water with a Potter-Elvehjem homogenizer and assayed for β-gal activity according to the manufacture's instructions. The activities were calibrated by subtracting the endogenous cellular β-gal activity in age-matched untreated controls at each time point and expressed as activity per total protein (milliunit/mg). Protein concentrations were determined with the BCA protein assay reagent (Pierce, Rockford, IL, USA) using bovine serum albumin (BSA) as standard.

Quantitative PCR

The mRNA level of the LacZ gene was quantified by quantitative reverse-transcription (RT)-PCR. Total RNA was extracted from the whole brain of recipients using the RNeasy lipid tissue kit (Qiagen) following the manufacturer's instructions including DNase I treatment. The RT reaction was performed using the SuperScript first-strand synthesis system (Invitrogen) with an oligo(dT) primer. The TaqMan probe and primers specific for the LacZ gene were used as previously described [20]. The quantities of LacZ gene were normalized with endogenous control β-actin gene to account for the variability in the initial concentrations and qualities of template. β-Actin cDNA was estimated by quantitative RT-PCR with SYBR Green dye. The primers used to detect mouse β-actin cDNA designed by Primer Express software (ABI, Tokyo, Japan) were 5′-tattggcaacgagcggttc-3′ (forward primer) and 5′-tggatgccacaggattccat-3′ (reverse primer, amplicon length of 78 bp). The PCR reaction was carried out in an ABI Prism 7700 sequence detection system under conditions described previously [1]. Each standard and sample was duplicated. The absence of nonspecific amplification including primer dimer formation was confirmed by dissociation curve analysis immediately after the reaction.

To quantify the amount of integrated provirus DNA in the recipients' brains, quantitative PCR for the LacZ gene was performed as previously described [1].

Analysis of tissue distribution of vector

Multiple tissues were harvested from wild-type recipients and their mothers at analysis. Genomic DNA was extracted using the DNeasy tissue kit (Qiagen). The primers used to detect the LacZ gene and the mouse β-actin gene were the same as that for real-time PCR previously described [1] (amplicon lengths of 122 and 77 bp, respectively). Genomic DNA (300 ng) was added to AmpliTaq Gold Master Mix (ABI) containing 0.2 μM of each primer in a final volume of 50 μl. The PCR reactions were carried out as denaturation and activation at 95 °C for 5 min, followed by 40 cycles (for the LacZ gene) or 30 cycles (for the β-actin gene) of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min. Ten microliters of each PCR product were electrophoresed on a 3.5% agarose gel and stained with ethidium bromide. To determine the sensitivity of the PCR condition for the LacZ gene, a standard was generated from the genomic DNA of the brain of heterozygous *Bmp4*^{lacZneo}, a LacZ knock-in mouse strain containing a single copy of the LacZ gene [21,22], serially diluted (10^{-1} to 10^{-5}) with normal mouse genomic DNA. The sensitivity of the LacZ PCR is about 1 copy of LacZ gene in 10^4 genomes.

In one animal (recipient at 15.5 months, animal-1 in Figure 6), tissues were harvested and divided into two parts. One part was used for PCR analysis as described above. The other part of the tissues was embedded in OCT compound. Serial cryosections were made at a thickness of 10 μm. Blood was obtained from a peripheral vessel and bone marrow cells were collected from the femur, tibia and pelvis. Cell suspension of the spleen was prepared by manual dissociation. Mononuclear cells were isolated from the blood, bone marrow and the spleen by Ficcoll-Conray (IBL, Fujioka, Japan) density gradient separation. Smears of mononuclear cells were prepared on MAS-coated slide glasses (Matsunami, Osaka, Japan) and allowed to air dry for 30 min at RT. Cryosections and smears were fixed with 0.25% glutaraldehyde in PBS for 10 min, incubated with X-gal staining solution overnight at 37 °C, and counterstained with H&E and nuclear fast red, respectively. The rest of the mononuclear cells from the bone marrow and the spleen were used for PCR analysis.

Results

Efficient gene transfer into embryonic brain under optimized conditions

It is known that the addition of positively charged compounds, such as Polybrene, during infection facilitates the infection of cells by retrovirus *in vitro*. To determine the satisfactory concentration of Polybrene *in utero*, concentrated LHCAL supplemented with different concentrations of Polybrene (at a final concentration of 0, 40, 80 or 160 μg/ml) was injected into the cerebral lateral ventricles of mouse embryos at E12. The gene transduction efficiency was evaluated by the contents of integrated provirus in the recipients' brains at 5 days after injection (E17). Quantitative TaqMan-PCR for the LacZ gene was used to quantify provirus DNA. Results showed that the transduction level increased with increasing Polybrene concentrations (Figure 1a). There was a linear correlation ($r = 0.999$) between transduction level and Polybrene concentration in the range 0–80 μg/ml. The level of gene transduction reached a peak at a concentration of 80 μg/ml. However, further increment of the concentration to 160 μg/ml did not increase the transduction efficiency.

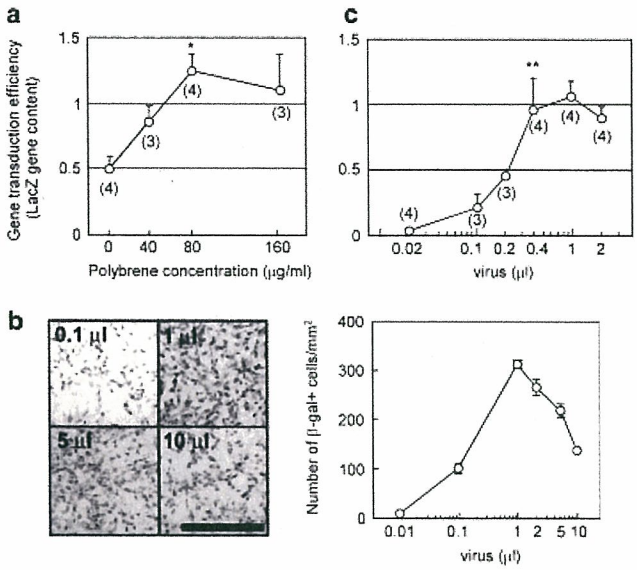

Figure 1. Optimization of transduction conditions. (a) Gene transduction efficiencies when LHCAL supplemented with various concentrations of Polybrene were injected. *$P = 0.02$ and $P = 0.72$, when transduction efficiency on Polybrene concentration of 80 μg/ml compared with that on 0 and 160 μg/ml, respectively (Mann-Whitney test). (b) NIH/3T3 cells grown in 24-well plates were infected with various titers of concentrated LHCAL and the transduction efficiencies were evaluated by X-gal staining. Left: Representative photographs of X-gal-stained NIH/3T3 cells. The volumes of virus stock used per well are indicated. Right: Number of β-gal+ cells per mm² ($n = 3$). (c) Gene transduction efficiencies when various amounts (μl) of concentrated virus were injected. **$P = 0.77$ and $P = 0.39$, when transduction efficiency on a volume of 0.4 μl/embryo compared with that on 1 or 2 μl/embryo, respectively (Mann-Whitney test). The data are presented as mean ± SE, the numbers in parentheses are numbers of animals studied. Scale bar: 1 mm

An *in vitro* study using the concentrated LHCAL to infect NIH/3T3 cells showed that excessive titers of viruses do not improve, and even reduce, the infection efficiency (Figure 1b). This result suggests that some factors in virus stock may inhibit infection, especially in high multiplicity of infection (MOI) condition. To determine the satisfactory doses of virus to achieve the highest transduction efficacy *in utero*, serially diluted virus stock (supplemented with 80 μg/ml of Polybrene) was injected into the cerebral ventricles of mouse embryos at E12 and transduction efficiencies were evaluated by the contents of provirus in the recipients' brains as above. Results showed that transduction levels increased with increasing virus doses in the range 0.02–0.4 μl of concentrated LHCAL per embryo, then reached a plateau when 0.4 μl of virus stock was injected. Further increasing of virus to 2 μl did not increase the transduction level (Figure 1c).

The results showed that the satisfactory concentration of Polybrene added to virus is 80 μg/ml and 0.4 μl of concentrated LHCAL (1.6×10^8 particles per embryo) is the minimal dose to achieve the highest infection efficiency *in utero*. This condition was used throughout the following study.

Widespread gene transduction to the CNS by retrovirus *in utero*

To investigate the pattern of gene transduction to the CNS by retrovirus *in utero*, we injected LHCAL to the lateral ventricles of mouse embryos at E12. At E12, many cells lining the ventricular zone are phosphorylated-histone-H3 positive (Figure 2a), indicating they are undergoing active proliferation. This permits the possibility of efficient transduction of these cells, since integration of murine leukemia virus (MLV) DNA into nuclear DNA of cells depends on mitosis and active division of target cells is the prerequisite of highly efficient gene transduction.

Seventy-one out of 76 embryos (93.4%) who received *in utero* injection of LHCAL were alive when analyzed at E17. For postnatal analysis, the recipients were allowed

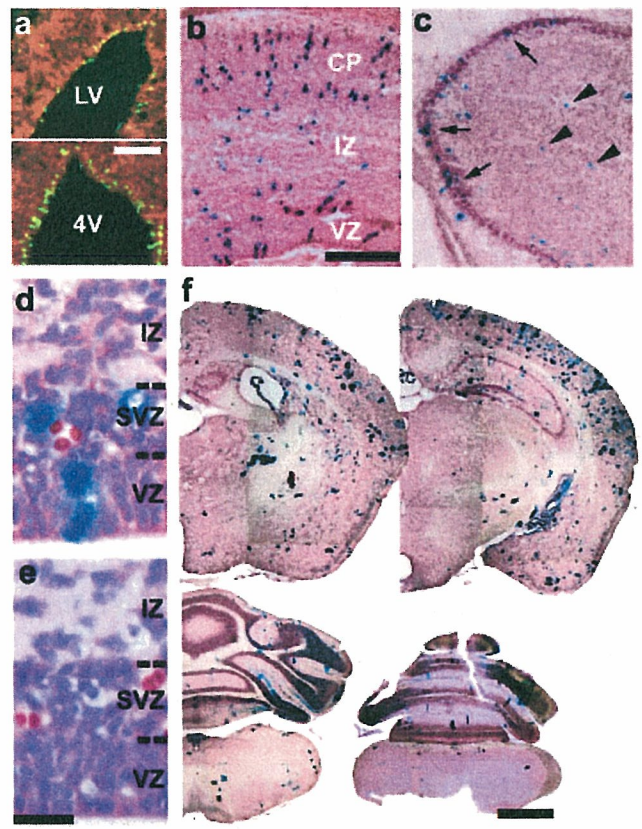

Figure 2. Widespread CNS gene transduction after LHCAL injection. (a) Immunofluorescence of brain sections of mouse embryos at E12 stained with anti-phosphorylated histone H3, a mitosis-specific marker. Many ventricular zone cells were phosphorylated histone H3 positive (green) indicating they were in active proliferation (red: propidium iodide nuclear counterstaining). (b–d, f) X-Gal-stained coronal brain sections from the mice that received LHCAL at E12. (b) Cerebral cortex at E17. (c) Cerebellar anlage at E17. Arrowheads, β-gal+ cells inside cerebellum. Arrows, β-gal+ external granular cells. (d, e) Microscopic photographs of paraffin sections of cerebral cortex from an embryo that received LHCAL injection (d) and the untreated litter-mate (e) at E17. (f) Brain sections at P30 listed rostral to caudal. 4V, 4th ventricle; CP, cortical plate; IZ, intermediate zone; LV, lateral ventricle; SVZ, subventricular zone; VZ, ventricular zone. Scale bar: 20 μm in (d, e); 100 μm in (a); 200 μm in (b, c); 1 mm in (f)

to be delivered normally and 80% (36/45) of them were alive at postnatal day 3–4 (P3-4). All pups alive at P3-4 ($n = 36$) survived to weaning and developed normally without noticeable behavioral abnormality. These data indicate that approximately 7% of recipients were dead before E17, and another 13% of recipients died between E17 and P3. The final survival rate of the recipients (80%) was similar to that in the normal development of untreated mice at E19 (67–100%, average 84%) [23], suggesting that the injection did not significantly affect survival rate in these mice. No tumors or other anatomical abnormalities were observed in the recipients' brains.

Efficient gene transfer to the CNS was observed in all recipients examined by X-gal histochemical staining at various time points ($n = 8$ at E17; $n = 26$ at 1–14 months). β-Gal+ cells were diffusely distributed throughout the brain of the recipients when analyzed at 5 days after injection (E17) (Figures 2b and 2c). In the cerebellum, in addition to scattered β-gal+ cells, some external granular cells, the progenitors of the granule cells, were also transduced (Figure 2c, arrows). No obvious difference was observed in the density and the morphology of the cells in the ventricular and subventricular zone of the cerebral cortex between the infected mice and their untreated littermates at E17 (Figures 2d and 2e), suggesting that the injection of virus and Polybrene under the condition used in this study has no significant cytotoxicity on the developing brain cells. At P30, when the migration and differentiation of neural cells are almost completed in the mouse brain, many β-gal+ cells were identified in most regions of the brain although regional differences in the intensities of transduction could be observed (Figure 2f). Most intensive sites of transduction were the cerebral cortex and cerebellar granular layers. A moderate number of transduced cells were distributed in the basal ganglia, hippocampal formation, thalamus and the cerebral and cerebellar white matter. A relatively smaller number of transduced cells were distributed in the brain stem. No positive signal was observed in untreated control brains.

Characterization of transduced neural cells

The cell types of transduced neural cells were identified by their morphology and anatomical locations. Immunohistochemistry against β-gal was used to identify the transduced neural cells. We found that the immunostaining gave more detailed and sensitive detection of positive cells and the distribution pattern of positive cells in the immunostaining was similar to that in X-gal staining.

Many β-gal-immunoreactive neurons with relatively large cell bodies and long processes were distributed in the cerebral cortex, basal ganglia and the hippocampal formation (Figures 3a and 3b). In the cerebellum, numerous immunoreactive granule cells packed in granular layers and some Bergmann glia and Purkinje cells extended processes to the molecular layer (Figure 3c).

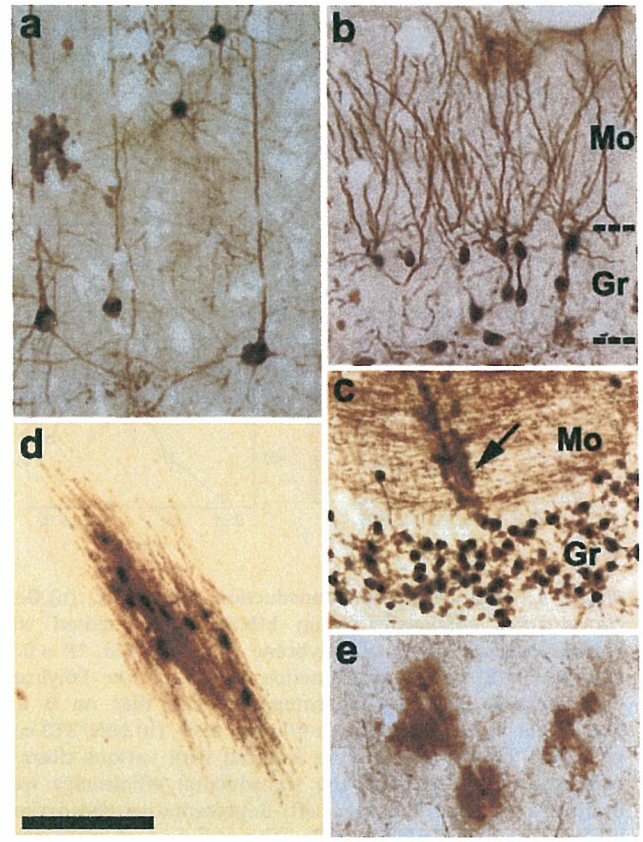

Figure 3. Morphological identification of cell types of transduced cells. (a) β-Gal-immunoreactive cortical neurons. (b) β-Gal-immunoreactive granule neurons in the dentate gyrus of the hippocampal formation. (c) Numerous cerebellar granule neurons were β-gal-immunoreactive. Note the parallel fibers of granule cells in the molecular layer were also immunoreactive. Arrow, an immunoreactive Bergmann glia. (d) A cluster of immunoreactive oligodendrocytes in the white matter of cerebral cortex. Note many immunoreactive longitudinal processes arrayed in parallel describing the myelin segments supported by transduced oligodendrocytes. (e) Some immunoreactive protoplasmic astrocytes in the cerebral cortex. Gr, granular layer; Mo, molecular layer. Scale bar: 100 μm in (a–e)

Transduced oligodendrocytes were distributed both in the gray and white matter throughout the brain and could often be observed as clusters. The myelinating oligodendrocytes had many immunoreactive longitudinal processes arrayed in parallel, connecting to the cell soma by short branches (Figure 3d). The longitudinal processes were presumably the inner and outer cytoplasmic tongues of the myelin sheaths and describe the myelin segments supported by transduced oligodendrocytes. Most transduced astrocytes were distributed in the gray matter having protoplasmic morphologies with highly branched feather-like processes (Figure 3e). Frequently, immunoreactive processes terminated as an end foot on a blood vessel could be seen. The proportion of transduced neural cells in some brain regions is shown in Table 1.

The morphological identification of cell types was further confirmed by double immunofluorescence for β-gal and cell-type-specific markers (NeuN, a neuronal marker; GFAP, an astroglial marker; GST-π [24] and

Table 1. Proportion of transduced neural cells in various brain regions

Cell type	Cerebral cortex[a]	Hippocampal formation	Diencephalon	Brain stem	Cerebellar white matter
Neuron	31.2 ± 1.6% (700)	54.6 ± 2.4% (823)	9.9 ± 1.7% (98)	2.2 ± 1.4% (9)	0% (0)
Astrocyte	53.9 ± 0.7% (1199)	40.2 ± 3.7% (614)	54.3 ± 2.9% (499)	43.3 ± 2.7% (158)	31.0 ± 1.2% (194)
Oligodendrocyte	14.9 ± 1.5% (327)	5.2 ± 1.4% (74)	35.8 ± 3.7% (318)	54.5 ± 2.6% (199)	69.0 ± 1.2% (435)
Total	100% (2226)	100% (1511)	100% (915)	100% (366)	100% (629)

Data is presented as mean ± SE ($n = 3$). In parentheses are the sums of cell numbers counted from three animals.
[a]Including the cerebral white matter.

MBP, oligodendroglial markers) on the recipient brain sections (Figure 4). β-Gal-immunoreactive longitudinal processes of oligodendrocytes were also overlapped with MBP (data not shown).

Long-term gene transfer to the brain

Twenty-six recipients (aged from 1 to 14 months) were examined for long-term expression and the maintenance of transgene by histochemistry, enzymatic assay and PCR-based RNA/DNA analysis.

By X-gal staining, the general distribution patterns of β-gal+ cells in the transduced brains at 1, 3, 6, 9 and 14 months after birth were similar. Although there were some variations in the number of positive cells among animals, the overall transduction level was similar and did not exhibit the trend of decrement with time (Figure 5a). Quantitative assessment of the β-gal activities in brain homogenates revealed that β-gal expression was constant throughout 14 months (Figure 5b, top). The LacZ transcript was also monitored by quantitative RT-PCR using LacZ-gene-specific probe and primers. Similar to protein expression, the β-gal mRNA expression also persisted to at least 14 months after birth (Figure 5b, middle). Brain tissues were also examined for the presence of transduced LacZ gene by quantitative PCR and results showed that the contents of LacZ DNA (i.e. provirus DNA) in the brains were stably maintained for 14 months without decrement (Figure 5b, bottom).

Tissue distribution of viral vectors

To determine whether gene transduction by intraventricular administration of retrovirus was limited to the CNS, PCR analysis was performed on the genomic DNA extracted from different tissues of six recipients ($n = 3$ at E17; $n = 3$ at 15.5 months, females) to detect integrated provirus DNA (the LacZ gene). Results showed that, in addition to the brain, multiple tissues including the skin, heart, lung, liver, ovary and bone marrow mononuclear cells from the recipients contained the LacZ sequence, although the positive tissues were not consistent among animals (Figure 6). Kidneys were negative for the LacZ gene in all six recipients examined. To confirm the presence/expression of LacZ gene in these tissues, X-gal staining was performed on the sections of

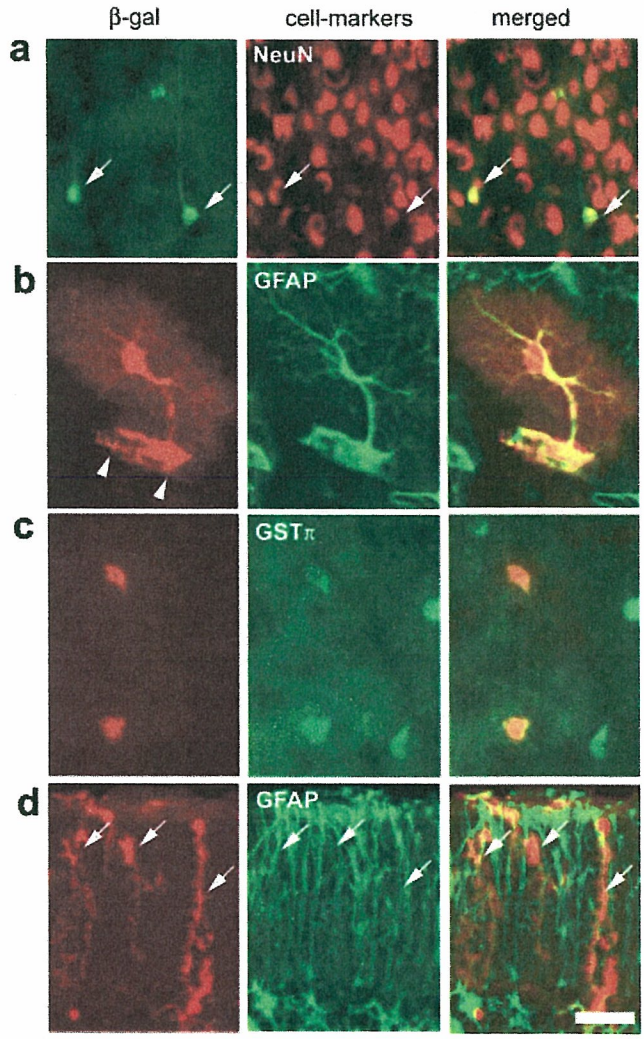

Figure 4. Cell-type identification of transduced cells by double immunofluorescence. (a) β-Gal-immunoreactive cells (green) in the cerebral cortex were NeuN+ (red), indicating they are neurons. (b) A β-gal-immunoreactive cell (red) in the cerebral cortex was GFAP+ (green), indicating it is an astrocyte. It has protoplasmic morphology and has a blood-vessel end foot (arrowheads). (c) β-Gal-immunoreactive cells (red) in the brain stem were immunoreactive for GST-π (green), an oligodendrocyte-associated enzyme, indicating they are oligodendroglia. (d) Some β-gal-immunoreactive cells (red) with processes extended to the molecular layer of the cerebellar cortex were GFAP+ (green), indicating they are Bergmann glia. Scale bar: 40 μm in (a, d); 20 μm in (b, c)

the heart, lung and liver and mononuclear cells of the bone marrow, spleen and the peripheral blood from one

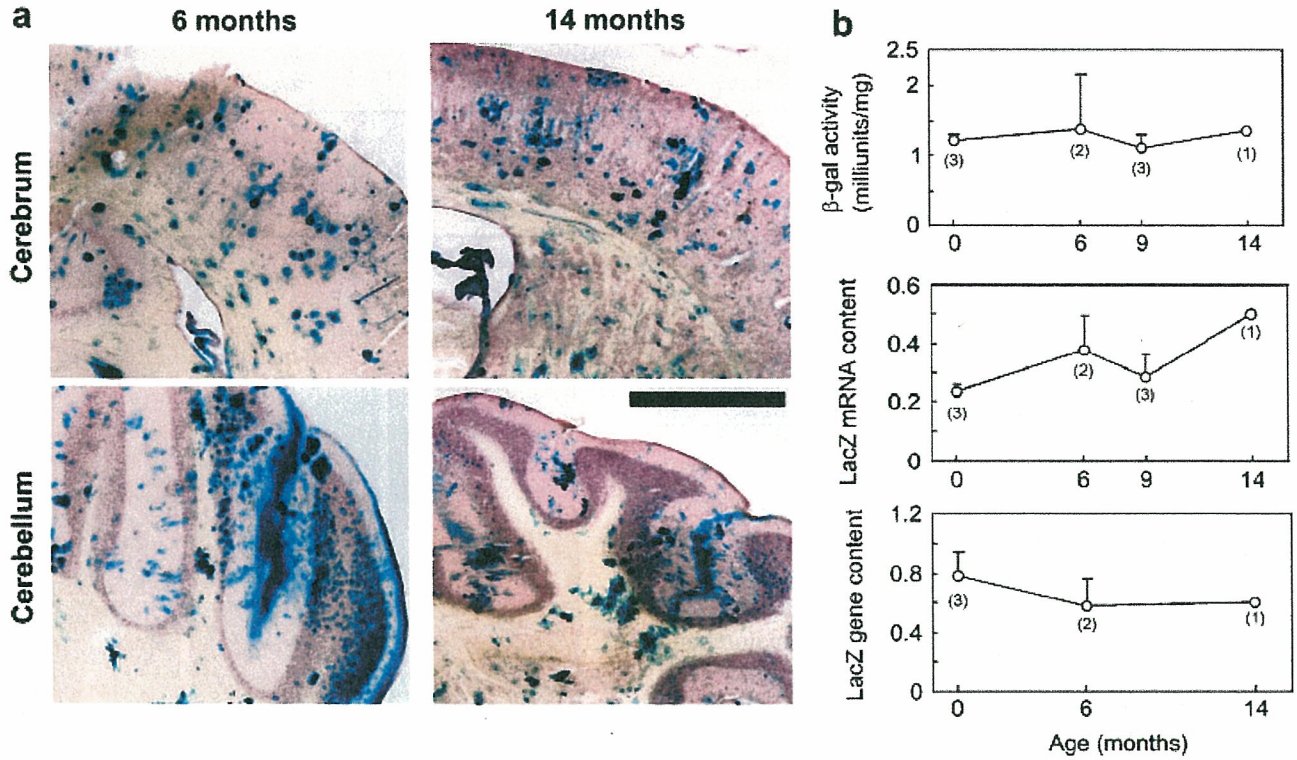

Figure 5. Long-term presence/expression of transgene in recipients' brains. (a) X-gal-stained coronal sections of the brain which received LHCAL at E12. (b) Quantitative analysis of the level of transgene and its expression in the brain at various time points. Top: β-gal enzyme activities in the brain. Middle: LacZ mRNA expression in the brain assessed by quantitative RT-PCR. Bottom: Amount of provirus DNA in the brain assessed by quantitative PCR for the LacZ gene. The numbers in parentheses are numbers of animals studied. Scale bar: 1 mm

recipient (15.5 months, animal-1 in Figure 6). β-Gal+ cells were detected from the heart, liver and bone marrow cells although the transduction level was much lower than that in the brain (e.g., 0–4β-gal+ cells per longitudinal section of the heart) (data not shown). These observations indicate that vectors injected into the cerebral ventricles spread, probably through fetal circulation, to multiple organs of recipients. To further determine whether transplacental transfer of vector from the fetus to the mother occurs, tissues from two mothers whose embryos received intraventricular injection of LHCAL were also tested by LacZ-PCR at 17 days gestation. Several organs including the heart, lung, liver and the kidney from the mothers were positive for the LacZ sequence (Figure 6).

Observations in twitcher mouse brain that received LHCAL *in utero*

The goal of our study is the application of intrauterine gene therapy to genetic neurodegenerative disorders such as Krabbe disease. The efficient gene transfer into neural cells including oligodendrocytes suggested the possible utility of prenatal gene transfer by retrovirus in the treatment of this kind of disease. As a preliminary study of the next challenge of therapeutic study using this approach in animal models of Krabbe disease, LHCAL was injected into the mouse embryos of pregnant twitcher heterozygotes (twi/+) at gestational day 12. The survival rate of the recipients to weaning was 63.3% (19/30), which is similar to that in the normal C57BL/6 strain (19/30 vs. 36/45, $P = 0.12$, Fisher's exact test). Four homozygous twitcher mice were obtained and gene transduction to the brain was analyzed by β-gal immunohistochemistry ($n = 2$ at P20; $n = 1$ at P30; and $n = 1$ at P41).

Efficient introduction of the LacZ gene into the brain was achieved in all recipient twitcher mice and their unaffected littermates examined (Figures 7a and 7b). The distribution pattern and the composition of transgene positive cells in the twitcher mice were similar to that in the normal mouse strain described above. Consistent with earlier studies [25,26], morphological alterations including enlarged somas and swellings in processes were recognized in some of transduced oligodendrocytes in the twitcher mice even at P20 (Figure 7d). At P30, many transduced oligodendrocytes in twitchers showed these morphological alterations (88.4% (191/216 cells) in the cerebrum; 82.6% (138/167) in the brain stem; and 79.7% (106/133) in the cerebellar white matter). At P41, abnormal swellings in processes further progressed in number and size generally and unstained vacuoles could be frequently seen in the swellings (Figures 7e and 7f). The changes were shown in most, but not all, transduced oligodendrocytes. Immunoreactive 'Myelin segments', however, were still relatively preserved in

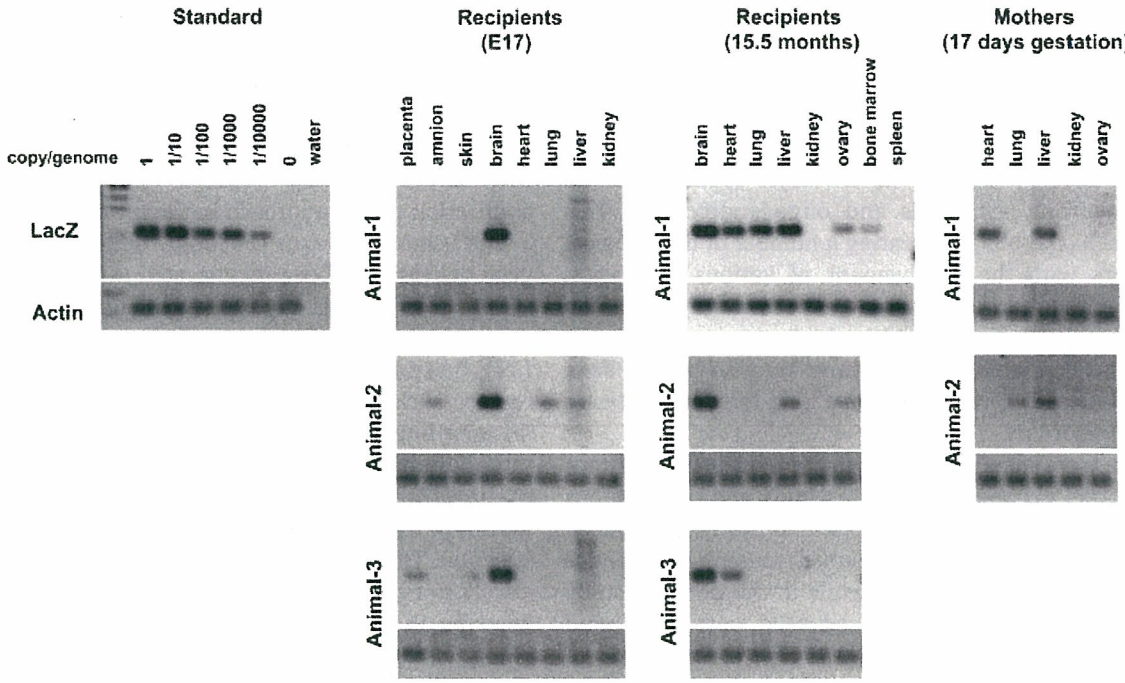

Figure 6. PCR analysis of tissue distribution of viral vector. LacZ-specific PCR (amplicon length of 122 bp) was used to detect provirus DNA in tissues from LHCAL recipients ($n = 3$ at E17; $n = 3$ at 15.5 months of age) and mothers ($n = 2$ at 17 days gestation). Many tissues were positive for the LacZ sequence in addition to the brain

many oligodendrocytes, despite severe abnormalities of the soma and processes (Figure 7f).

Discussion

This study demonstrated that direct intraventricular injection of retrovirus *in utero* is a useful strategy to achieve efficient and persistent gene transfer to the brain before birth.

In order to obtain high infection efficiency *in vivo*, retrovirus was concentrated and the transduction conditions were optimized. Although Polybrene has often been used in retrovirus-mediated *in utero* gene transfer to facilitate infection, the concentrations used vary significantly among laboratories [5–9]. Our results showed that the optimal concentration of Polybrene is 80 μg/ml (160 ng per embryo) to achieve the highest transduction efficiency without noticeable toxicity in the embryonic mouse brain. We also showed that excessive titer of retrovirus could not increase or even inhibit the transduction both *in vitro* and *in vivo*. A similar phenomenon was also seen in another *in utero* gene therapy study [3], in that 1000-fold increased titers of retrovirus led to only 2–8-fold increment of transduction efficiency. The discrepancy between the end-point titer and transduction efficiency has been documented by Forestell *et al.* previously [27], and the authors suggested that, in high MOI condition, inhibitors (non-transducing viral particles and free viral envelope proteins) present in the virus stock would compete with transducing virions for receptor-binding sites on the target cells and reduce transduction efficiency.

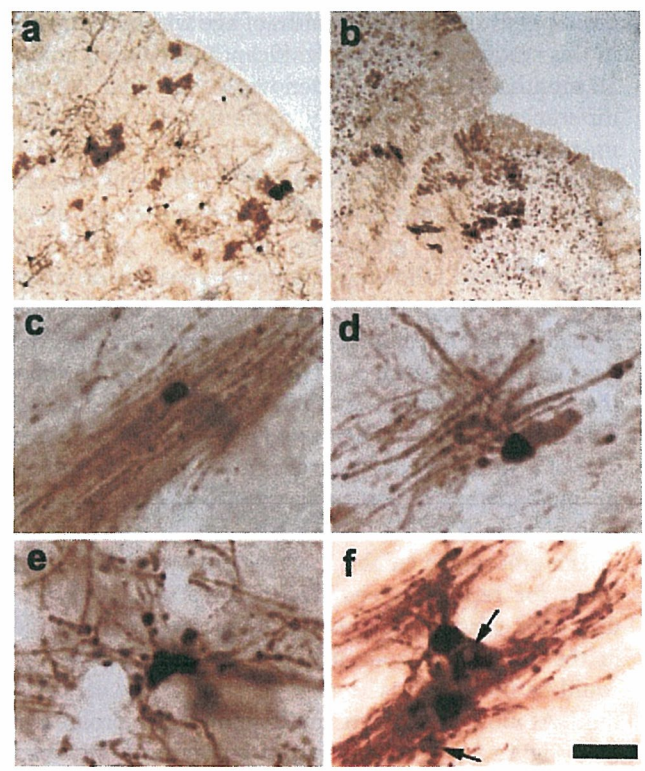

Figure 7. Efficient transduction to oligodendrocytes in the twitcher brain after LHCAL injection. Anti-β-gal immunohistochemistry of the brain sections from the twitcher mice which received LHCAL at E12. (a, b) Overview of widespread gene transduction to the twitcher brain at P30. (a) Cerebral cortex. (b) Cerebellar cortex. (c–f) High-magnitude view of immunoreactive oligodendrocytes from control and the twitcher mice. (c) Wild-type littermate at P41. (d) Twitcher at P20. (e, f) Twitcher at P41. Arrows, unstained vacuoles in the swellings. Scale bar: 200 μm in (a, b); 20 μm in (c–f)

Our results suggest that the satisfactory titer should be determined when retrovirus is used *in utero*.

After *in utero* gene transfer, transgene-expressing neural cells were observed throughout the brain by X-gal histochemistry and immunohistochemistry. Efficient gene transfer was achieved in major types of neural cells (neurons, astrocytes and oligodendrocytes). This suggests a possibility of treating a number of neurological disorders with the involvement of various cell types. Another important finding is the permanent expression of vector-encoded gene within the brain suggesting that retrovirus-mediated *in utero* gene transfer might be a useful approach toward achieving long-term correction of genetic disorders.

There were some differences in the transduction pattern in the brain between the present study and our previous study using adenovirus [1]: (i) In this study, transduced neural cells were widely dispersed spanning the entire depth of the cerebral cortex, while in the previous study using adenovirus the transduced cells were distributed predominantly in certain layers of the cortex (e.g., distributed in layers VI-VI when adenovirus was injected at E12). (ii) Both neurons and glia were transduced in similar proportions in this study, while the majority of the transduced cells were neurons when adenovirus was used. (iii) The levels of transgene and its expression in the brain persisted up to 14 months of age without decrement in this study, while these significantly decreased at about 10 months of age when adenovirus was used. All these differences are most likely attributed to the different properties of the two types of viruses regarding the capacity of integration. In contrast to retrovirus that could permanently transmit the virus genome to all descent cells, episomally presenting adenovirus genome was rapidly diluted following sequential divisions of infected progenitor cells, thus only the cells undergoing few divisions after infection could contain enough adenovirus genomes and produce detectable level of β-gal. Although the powerful infection capability of adenovirus makes it a useful tool to study *in utero* gene delivery routes and the role of a specific gene, from the therapeutic point of view for most genetic diseases that require lifetime correction, retrovirus may be more desirable.

The embryonic cerebral ventricular system is an ideal route for retrovirus-mediated *in utero* gene transfer to the brain. Firstly, multipotent neural stem cells resident in the ventricular zone at early stages of development could be infected by virions presenting in the ventricular fluid. As most of them are undergoing active proliferation (see Figure 2a), efficient gene transfer with MLV-based retrovirus which requires mitosis for integration would occur. Secondly, most neural cells in the mature brain arise from small numbers of neural stem cells by sequential divisions. Thus, even if small numbers of primitive stem cells were transduced *in utero*, expansion of their progeny could lead to significant amplification of the transgene (e.g., see significant proliferation of β-gal+ cerebellar granule neurons from small number of transduced external granular cells; Figures 2c, 2f and 3c). Thirdly, as the ventricular system is a relatively closed narrow cavity, the ventricular zone could be infected effectively by a relatively small dose of retrovirus. Finally, the cerebral ventricle of a human fetus is easily identified and targeted under ultrasound guidance at an early stage of gestation providing the technical feasibility of clinical application of this approach in the future.

The use of retrovirus (including lentivirus) for *in utero* gene delivery has been investigated in a number of studies previously. Viral supernatant or retroviral producing cells were introduced into rats [28], mice [29–31], dogs [32], sheep [2,3] and monkeys [4,33,34] through various routes, such as intraperitoneal, intrahepatic, intrapulmonary, intramuscular, intra-amniotic and intraplacental routes, prenatally. Transduced tissues varied among these studies. This probably depended on the differences in the species of animal models used, target sites and the time of injection. The injection into certain tissues resulted in relatively site-specific transduction [31,33]. Intraperitoneal injection, in general, resulted in the transduction of nearly all organs and the hematopoietic cells [2–4,32]. Porada *et al.* [2] reported that the expression of transgene in hematopoietic cells persisted more than 5 years in sheep that received a single *in utero* intraperitoneal injection of retrovirus. No adverse effects to the fetuses or the mothers were reported in these previous studies. These investigations indicated that *in utero* introduction of retrovirus vector could be a relatively safe and effective method to achieve persistent transduction of exogenous genes. In addition, Tarantal *et al.* [4] demonstrated that intraperitoneal injection of HIV-1-derived lentivirus resulted in a significantly greater transduction frequency in hematopoietic progenitor cells when compared with a similar titer of MLV-based vectors. In the present study, although MLV-based retrovirus was used, we expect that lentivirus may provide a greater level of gene delivery since it could integrate into both dividing and non-dividing cells.

Although the primary target of this study is the transduction of the CNS, multiple organs were also transduced in the recipients. The wide distribution of the retroviral vector throughout the body suggests that some virions accessed the fetal circulation. We speculate that the blood-cerebrospinal fluid (CSF) barrier (mainly constituted with tight junctions connecting the apical regions of the epithelium of the choroid plexus) may not be fully formed or matured at the injection time and this may allow the transport of virions from the CSF into the circulation. Gene transduction to multiple systems such as the heart, lung, liver and the bone marrow other than the brain may be helpful to the treatment of storage diseases since almost all storage diseases with CNS involvement also affect the peripheral nervous system and/or other non-nervous systems. However, the levels of transduction in these organs were very low in this study and whether it could provide therapeutic benefit is unclear at this time.

The presence of vector sequence in the ovaries of the recipients in this study is of concern regarding the safety of this approach, although germ-line transmission in these animals was not determined. Many previous

studies also reported that vector DNA was detected within the gonads of the mouse, dog, sheep and monkey after *in utero* administration of retroviral vectors [2–4,31–33]. Breeding studies and PCR analysis of purified sperm cells, however, showed no evidence of the transduction of germ line in the sheep [2,3]. In our study, vector DNA was also detected in the maternal tissues, suggesting the possibility of transplacental transport of vectors into the maternal circulation. Previous studies of *in utero* gene delivery using retrovirus reported that provirus DNA was detected in maternal tissues of the sheep [2], but not the monkey [4,33], and the reason for these different findings was still not known. One possible explanation may be the structural differences of the placenta among species (mouse, sheep and monkey) which may determine whether or not virions could cross the placenta.

Although we did not find evidence of tumor formation on semi-serial sections from the recipients' brains in this study, it is difficult to exclude the possibility of the presence of tumors of small sizes. Since retroviruses including lentivirus integrate into the host genome in a random manner, there are potential risks of influence on the activity of adjacent genes that could lead to tumor formation or developmental aberrations. This concern will be resolved eventually with the development of a viral vector that enables site-specific integration. Although our study suggests the potential utility of retrovirus-mediated *in utero* gene transfer via the intraventricular route for the treatment of genetic neurological disorders, further extensive and rigorous studies, especially using non-human primate models, will be required to explore the safety and feasibility of this approach in human fetuses.

At present, treatment for Krabbe disease is limited to hematopoietic stem cell transplantation. Neurological manifestations of the patients could be significantly improved following transplantation [35,36]. On the other hand, animal experiments that introduced the GALC gene into twitcher mice and cultured cells by virus vectors or transgenic technology showed various degrees of correction of biochemical, pathological and clinical phenotypes suggesting the potential usefulness of gene therapy strategy in GLD [19,37–39]. The previous virus-mediated gene therapy studies in twitcher mice [19,40] have provided important points: (i) Gene transfer must be initiated from an early stage of disease course. (ii) Enzyme supplied to the brain should be in sufficient levels. (iii) Compared to other models of storage disease, the twitcher mice may be more difficult to treat because of the rapidly progressed deterioration of the nervous system. *In utero* gene therapy has been considered as an alternative approach to this disease [12,40,41] and viral vectors encoding reporter genes were introduced into the fetus of animal models through an intrahepatic route. Although multiple tissues were transduced in these studies, gene transfer to the brain cells was relatively limited [40,41]. Development of a gene delivery system that could achieve more global, sustained and regulated expression of GALC in brain cells may be needed. Our study demonstrates that intrauterine injection of retrovirus via cerebral ventricles could achieve efficient transfer and persistent expression of exogenous gene throughout the brain. Sufficient gene delivery to oligodendrocytes and the other cell types achieved in the twitcher mice is encouraging and suggests the potential utility of this method in treating GLD. Future investigations will be conducted to explore whether introduction of the GALC gene into the twitcher brain *in utero* could effectively correct the metabolic defect and prevent the morphological abnormality of the oligodendrocytes and the neurological symptoms.

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GALC transduction leads to morphological improvement of the twitcher oligodendrocytes in vivo

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Abstract

Globoid cell leukodystrophy (GLD, Krabbe disease) is a severe demyelinating disease caused by a genetic defect of β-galactocerebrosidase (GALC). To date treatment to GLD is limited to hematopoietic stem cell transplantation. Experimental approaches by means of gene therapy in twitcher mouse, an authentic murine model of human GLD, showed significant but only marginal improvements of the disease. To clarify whether the introduction of GALC could provide beneficial effects on the oligodendrocytes in GLD, we transduced twitcher oligodendrocytes by stereotactically injecting recombinant retrovirus encoding GALC-myc-tag fusion gene into the forebrain subventricular zone of neonatal twitcher mouse. In vivo effects of exogenous GALC on twitcher oligodendrocytes were studied histologically by combined immunostaining for the myc-epitope and the oligodendroglial specific marker, π form of glutathione-*S*-transferase, at around 40 days of age. We show here that GALC transduction led to dramatic morphological improvement of the twitcher oligodendrocytes comparing with those in untreated twitcher controls. This study provided direct in vivo evidence that GALC transduction could prevent or correct aberrant morphology of oligodendrocytes in GLD which may be closely related to the dysfunction and/or degeneration of oligodendrocytes and the demyelination in this disease.

Keywords: Krabbe disease; Twitcher; Galactocerebrosidase; Oligodendrocyte; Gene therapy; Retrovirus vector

Introduction

Globoid cell leukodystrophy (GLD, Krabbe disease) is a demyelinating disease caused by a genetic deficiency in the activity of a lysosomal enzyme, β-galactocerebrosidase (GALC, EC 3.2.1.46) [1]. In infantile form of this disease, the clinical symptoms occur soon after birth, progress rapidly and most patients die before 2 years of age. The rapid deterioration of the nervous system is postulated to be the result of the dysfunction and/or degeneration of myelin-forming cells (oligodendrocytes and Schwann cells) caused by an accumulation of galactosylsphingosine (psychosine), a cytotoxic metabolite and one of the substrates of GALC [2,3]. To date, there is no effective treatment to this disease except hematopoietic stem cell transplantation [4].

Twitcher mouse is a naturally occurring genetically authentic murine model of human GLD [5]. Deficiency in the activity of GALC is caused by a nonsense mutation at the coding region of GALC gene [6]. The clinical symptoms of the twitcher mouse include failure to gain weight, tremor, and progressive paralysis of hind limbs and early death around postnatal day 40 (P40). Similar to human GLD, the characteristic pathology in the central and peripheral nervous system (CNS and PNS) of the twitcher mouse is severe demyelination, infiltration

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of periodic acid-Schiff (PAS)-positive macrophages, and gliosis. In the brain, oligodendrocytes show morphological alterations and are gradually depleted by apoptotic death [7,8].

The treatments to GLD, like those for most of the other lysosomal storage diseases, are to deliver the deficient enzyme to the cells affected by the storage. Bone marrow transplantation [9–11], neural stem cell transplantation [12], and vector-mediated gene therapy [13,14] in the twitcher mouse showed various degrees of correction of the biochemical, pathological, and clinical phenotypes of the disease. The level of GALC activity appeared responsible for the therapeutic effects. So far, little direct evidence showing that exogenous GALC enzyme was delivered to myelin-forming cells is available. Recent study [15] suggested that down-regulated immune-related molecules in twitcher mouse following bone marrow transplantation may also contribute to clinicopathological improvements. Whether transduction of GALC into myelin-forming cells alone has therapeutic benefit will be crucial in treating GLD, especially by means of gene transfer. In vitro studies showed retrovirus-mediated transduction of GALC led to morphological normalization in cultured oligodendrocytes derived from twitcher mouse [16,17], however taken that the culture system may be different from the in vivo environments such as the absence of immunological environments which is considered to play important roles in the destruction of oligodendrocytes and demyelination in twitcher [18], it is important to clarify whether GALC transduction improves the morphology of the oligodendrocytes in GLD in vivo.

In this study, we introduced human GALC cDNA tagged with myc-epitope into a small number of oligodendrocytes in the twitcher brain by stereotactic injection of retrovirus into the subventricular zone (SVZ) at birth. By combined immunostaining for the myc-tag and the oligodendroglial specific marker, π form of glutathione-*S*-transferase (GST-π), we clearly show that GALC transduction led to dramatic morphological improvement of the twitcher oligodendrocytes comparing with those in untreated twitcher controls. The results provided direct evidence that GALC transduction could prevent or correct aberrant morphology of oligodendrocytes in GLD in vivo which may be closely related to the degeneration of oligodendrocytes and the demyelination process in this disease.

Materials and methods

Plasmids

The full-length coding region of human GALC cDNA was cloned [13] and inserted into *Cla*I site of pBluescript II KS (Stratagene, La Jolla, CA). To assess the influence of ATG surrounding sequence to GALC expression level, the region around the initiation codon was removed using *Not*I (in +7 position of GALC) and *Sac*I (in the vector), and replaced by three different fragments generated from synthetic complementary oligonucleotides containing an additional *Cla*I site. The first fragment contains an ATG surrounding sequence initially reported [19,20] and named as "original ATG" in this study. The second fragment contains Kozak consensus sequence [21] named as "Kozak ATG," and the third fragment contains another in-frame ATG at 48 bp upstream to original ATG [22] and named as "1st ATG." The sequences of these fragments were shown in Fig. 1. These three GALC cDNA with different ATG surrounding sequence were excised from pBluescript II KS using *Cla*I and cloned into *Cla*I site of retrovirus

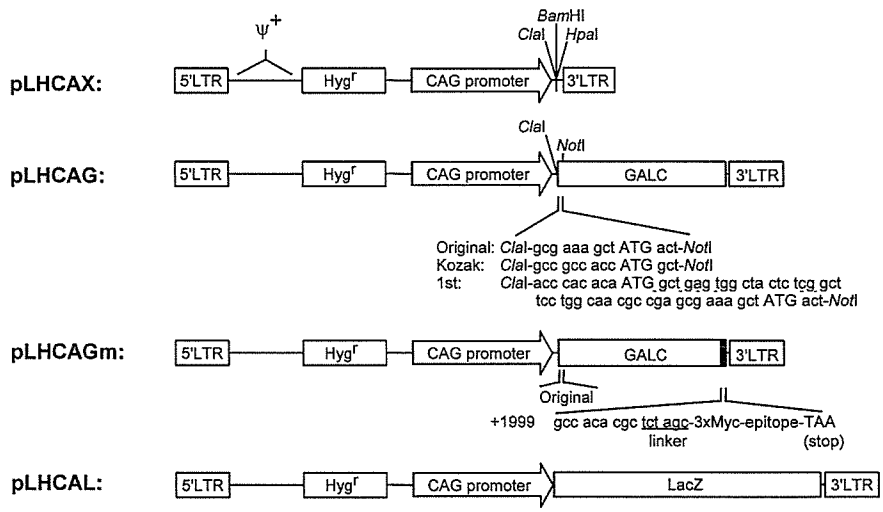

Fig. 1. Schematic representations of the retroviral constructs. See Materials and methods for details.

vector pLHCAX [23] to generate a series of retroviral vectors named pLHCAG (Fig. 1). In which, a hygromycin phosphotransferase gene was driven by the long terminal repeat (LTR) and GALC gene was driven by CAG promoter. To introduce myc-epitope, the stop codon (TAA, in +2008 position) of GALC (with "original ATG") was replaced with a unique *Xba*I site by polymerase chain reaction (PCR)-based mutagenesis. Complementary oligonucleotides encoding three tandem c-myc-epitope (EQKLISEEDL) followed by a stop codon (TAA) was cloned in-frame into *Xba*I site to create pLHCAGm (Fig. 1). In all ligated plasmids, the orientation and sequence of each insert was confirmed by DNA sequencing. A retrovirus vector expressing *Escherichia coli* β-galactosidase (β-gal), pLHCAL [23], generated by the insertion of LacZ gene between the *Bam*HI and *Hpa*I sites of pLHCAX was also used in this study (Fig. 1).

Transient expression study

Bosc23 and NIH/3T3 cells were grown in 12-well plates (NunC, Roskilde, Denmark) at 37 °C with 5% CO_2in Dulbecco's modified Eagle's medium (DMEM) with high glucose (4.5 g/L) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS). When approximately 95% confluent, the cells were transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendation (1.6 μg DNA:4 μl lipofectamine 2000 per well). Two days after transfection, the cells were washed with phosphate-buffered saline (PBS), harvested and assayed for GALC activity (as described below).

Virus preparation

To produce recombinant retrovirus, ecotropic packaging cell line ψMP34 [24] was transfected by calcium phosphate method (Cellphect transfection kit, Amersham, Piscataway, NJ) with pLHCAGm. After selection with 0.5 mg/ml hygromycin B (Wako, Osaka, Japan), drug-resistant colonies were isolated and a subclone (ψMP34/pLHCAGm#92) producing the highest titer of LHCAGm was used in this study. Retrovirus was concentrated according to the protocol reported by Bowles et al. [25] as described previously [23].

Viral titer assay and in vitro infection

For the assessment of infectious viral titers, NIH/3T3 target cells were grown in 24-well plates (for LHCAGm) or 6 cm dishes (for LHCAL) in DMEM supplemented with 10% FBS. When approximately 60% confluent, cells were infected with serially diluted viral stocks overnight at 37 °C in the presence of 8 μg/ml polybrene (Sigma, St. Louis, MO). Forty-eight hours after infection, cells were fixed and stained to detect transgene expressing cells. The cells infected with LHCAGm were fixed with 4% paraformaldehyde in PBS for 10 min on ice, and stained for myc-epitope using rabbit polyclonal antibody to myc-tag (MBL, Nagoya, Japan, 1:1500) as described in histochemical analysis section below. The cells infected with LHCAL were fixed with 0.25% glutaraldehyde in PBS and stained with X-gal solution (1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$ in PBS) overnight at 37 °C. The number of positively stained colonies was counted under a microscope and the titer was calculated as follow: cfu/ml = number of positive colonies/virus volume (ml). The titers of concentrated LHCAGm and LHCAL were 1.2×10^7 and 7.3×10^7 cfu/ml, respectively.

To evaluate intracellular activity of transduced GALC gene, spontaneously immortalized fibroblasts derived from twitcher mouse designated as Tw2 were infected with LHCAGm in the presence of 8 μg/ml polybrene and selected with 0.5 mg/ml hygromycin B. A drug-resistant subclone (Tw2/LHCAGm#11) with the highest GALC activity was expanded and used for the galactocerebroside (GalCer)-loading study.

Animals and virus injection

Breeding pairs of twitcher heterozygotes (C57BL/6J, twi/+) were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in our laboratory under standard housing condition. To determine transduction pattern of SVZ injection in the mouse, nine normal C57BL/6J mice received LHCAL injection at P0 were analyzed for gene transfer to the neural cells by X-gal staining (at P4) or immunohistochemistry for β-gal (at P30). Twenty twitcher mice were used in this study: 10 mice received LHCAGm injection and seven out of eight surviving recipients were used for histochemical ($n = 4$) or biochemical ($n = 3$) analysis at P35–40; three received LHCAL injection and used for histochemical analysis at P38–39 and seven untreated twitcher mice were taken as controls for histochemical ($n = 4$) or biochemical ($n = 3$) analysis at P39–40. In addition, six untreated normal mice were used as controls for histochemical ($n = 3$) or biochemical ($n = 3$) analysis at P40.

The procedure of stereotactic injection of retrovirus into SVZ was modified from that reported by previous study in neonatal rats [26]. Briefly, the injection was performed on the day of birth after DNA diagnosis of the twitcher mutation by PCR [6]. The newborn mice were anesthetized by placing on ice for a few minutes, placed in a stereotactic apparatus (Narishige, Tokyo, Japan) and kept cold with ice packs. Approximately 1.0 μl HBSS containing retrovirus with 0.01% Fast Green (Sigma) was injected unilaterally through a 30 G needle with a micro syringe (Ito, Shizuoka, Japan). Stereotactic

coordinates (related to the intersection of transverse and longitudinal cerebral fissure) were anterior 1.5 mm and lateral 1.5 mm, at a depth of 2 mm. All pups used in this study were recovered from cryoanesthetization by warming prior to being returned to their mother.

The Fast Green dye was used to verify that the virus was not injected into the lateral ventricle. In pilot studies using LHCAL in normal mice showed that when retrovirus was injected into the cerebral ventricles few cells could be transduced in any location of the brain. In one twitcher mouse received LHCAGm injection in this study, virus entered the lateral ventricle and few myc$^+$ cell was found in the brain and no further analysis was carried out on this animal.

GALC activity assay

GALC activity was assayed as previously described [27] using ^{3}H-labeled GalCer as substrate. The cells were washed with PBS, harvested, and sonicated for 30 s on ice. For tissues, the forebrain was homogenized in distilled water with Potter–Elvehjem homogenizer and sonicated briefly. The homogenate corresponding to about 100 μg of total proteins was used for the assay. Protein concentrations were determined with BCA protein assay reagent (Pierce, Rockford, IL) with bovine albumin as standard.

Galactocerebroside-loading study

Galactocerebroside (GalCer)-loading study was performed essentially as described by Kobayashi et al. [28,29]. Briefly, ^{3}H-labeled GalCer in chloroform:methanol (2:1, v/v) and phosphatidylserine in chloroform:methanol (95:5, Sigma) were dried in a sterilized tube and mixed with DMEM supplemented with 10% FBS by sonication for 30 min. Confluent Tw2, Tw2/LHCAGm#11, and NIH/3T3 cells grown in 3.5 cm dishes were incubated with 1.5 ml the medium containing 0.33 μg [^{3}H]GalCer (12,000 dpm)/ml and 5 μg phosphatidylserine/ml. After 4 days, the medium was removed and cells were harvested by trypsin digestion. Intracellular [^{3}H]GalCer and released [^{3}H]galactose in medium was extracted as described [29], dissolved in ACSII scintillation cocktail (Amersham) and the radioactivities were counted in a scintillation counter (LS6500, Beckman Instruments, Fullerton, CA). Hydrolysis of GalCer was expressed as a percentage of the released galactose, on the basis of the total incorporated GalCer (the sum of galactose in the medium and intracellularly accumulated GalCer).

Western blot

The confluent cells grown in 6 cm dishes were lysed with 300 μl sample buffer. After heating at 95 °C for 5 min, 5 μl of total cell lysates were electrophoresed on a 10% sodium dodecylsulfate (SDS)–polyacrylamide gel and blotted to a PVDF membrane (Novex, San Diego, CA). The membrane was reacted with rabbit polyclonal antibody to myc-tag (MBL, 1:1000) at 4 °C overnight and the signals of reactive proteins were detected by ECL plus reagents (Amersham).

PCR and Southern blot

Total cellular RNA was extracted from the brain homogenate of the recipients and untreated control mice using RNeasy lipid tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions including DNase I treatment. The reverse-transcription reaction was performed using the SuperScript first-strand synthesis system (Invitrogen) with oligo(dT) primer. Genomic DNA was extracted from brain homogenate from treated twitcher mice and untreated control mice by DNeasy tissue kit (Qiagen). The primers specific for human GALC cDNA were designed as 5′-TGGAA CCCATTCAGCAAAAG-3′ (forward primer) and 5′-CTGCTTAAAAAGAAATCTTTCGGAT-3′ (reverse primer, amplicon length of 559 bp). The forward and reverse primer located in exons 8 and 12, respectively, spanning more than 17 kb of introns in genome, and the last 5–6 nucleotides (underlined) in 3′-termini are completely different from the sequences of the mouse GALC cDNA. Genomic DNA (100 ng) or cDNA corresponding to 0.6 μg total RNA were added to AmpliTaq Gold Master Mix (ABI, Tokyo, Japan) containing 0.5 μM of each primer in a final volume of 50 μl. The PCR was carried out as denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 15 s and extension at 72 °C for 1 min. Under these conditions, no amplification occurs in mouse genomic DNA or cDNA.

Ten microliters of PCR products were electrophoresed on a 1.5% agarose gel, photographed after ethidium bromide staining and transferred to a nylon membrane. The membrane was hybridized with a [^{32}P]dCTP labeled human GALC probe (1.1 kb *Hin*dIII–*Sph*I fragment of GALC cDNA) and exposed to X-ray film (Fuji Photo Film, Tokyo, Japan).

Histochemical analysis

The mice were anesthetized and fixed with transcardial perfusion of chilled 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) at the time of analysis. Brains were removed and postfixed in the same fixative overnight at 4 °C and cryoprotected in 15% sucrose in 0.1 M PB. Consecutive coronal sections of the forebrain were made at a thickness of 20 μm using a cryostat.

For immunohistochemistry, the sections were incubated with 0.3% Triton X-100 in 0.1 M PBS (PBST) for overnight at 4 °C, then treated with 0.5% H_2O_2 in PBST

for 30 min at room temperature (RT) to eliminate endogenous peroxidase activity. After incubation with 5% normal goat serum (NGS) in PBST for 1 h at RT, the sections were incubated with primary antibody (rabbit anti-β-gal, 5′ → 3′, Boulder, CO, 1:500; rabbit anti-myc-tag, MBL, 1:500 or rabbit anti-GST-π, MBL, 1:20,000) diluted in 3% NGS/PBST for overnight at 4 °C. The sections were then incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) diluted 1:200 in 3% NGS/PBST for 1 h at RT, followed by the incubation for 30 min with avidin–biotin complex (ABC) reagent (ABC elite kit; Vector Laboratories) at RT. The peroxidase labeling was visualized with 3,3′-diaminobenzidine (DAB) substrate (Vector Laboratories) with or without nickel chloride.

For double immunostaining, after treated with Triton X-100 and H_2O_2 as above the sections were incubated in order with blocking reagents, primary antibody (mouse monoclonal anti-β-gal, Promega, Madison, WI; 1:1000 or mouse monoclonal anti-myc (9E10), Santa Cruz Biotechnology, Santa Cruz, CA, 1:1000), biotinylated anti-mouse IgG reagent and ABC reagent (Vector MOM peroxidase kit, Vector Laboratories) according to kit instructions except for incubation with primary antibody overnight at 4 °C. The 3-amino-9-ethylcarbazole (AEC, Sigma) was used to localize peroxidase and the sections were kept in PBS on ice. The immunoreactive cells in the cerebral cortex were viewed and photographed under light microscope (4× and 40× objective) connected with a color CCD camera (Olympus, Tokyo, Japan) to document the positions and morphologies of positive cells. Then sections were immersed in a graded ethanol series to eliminate AEC reaction product. After rehydrated and treated with 0.5% H_2O_2 to eliminate any residual peroxidase activity, the sections were incubated with rabbit polyclonal antibody to GST-π (MBL, 1:8000) for 5 days at 4 °C and labeled with ABC elite kit (Vector Laboratories) as described above, visualizing peroxidase with DAB-nickel (Vector Laboratories). The sections were dehydrated, cleared, and mounted.

The AEC reaction product was eliminated before the second immunostaining because cytoplasmic localization of β-gal or myc tagged GALC was largely overlapped with that of the second antigen GST-π and the dense AEC product appeared interfere with subsequent processes of immunostaining to detect GST-π. However, based on carefully documented anatomical locations including relation with nearest vessels and the morphologies of positive cells after immunostaining of the first antigen, the majority of doubly positive cells were easily identified after GST-π immunostaining (see Figs. 6G–J). Every third section at the levels corresponding to level 15–25 in the "Brain Maps: Structure of the Rat Brain" by Swanson [30] were used for morphological analysis of GST-π^+ cells and for untreated controls one section per animal at level 25 was used for the analysis. The morphology of the cells after GST-π staining was examined under microscope and photographed.

Immunofluorescence study

Tissues were prepared as described above and double immunofluorescence was performed as previously described [13]. The used primary antibodies were: mouse monoclonal anti-β-gal (Promega, 1:500), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; Dako, Glostrup, Denmark; 1:300), rabbit polyclonal anti-GST-π (MBL, 1:500) and rabbit polyclonal anti-myelin basic protein (MBP; Dako; 1:500). The secondary antibodies used were: rhodamine-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG (Cappel, Aurora, OH; 1:100). The sections were viewed under a fluorescence microscope and photographed using an AquaCosmos CCD camera (Hamamatsu Photonics, Hamamatsu, Japan).

Results

Determination of influence of ATG surrounding sequence to GALC activity

It is known that the sequence around the initiation codon in GALC gene is not optimal for translation according to Kozak rule [19–21]. Prior to the generation of recombinant retrovirus, to determine the sequence around initiation codon that gives rise to the most optimal expression of GALC, we generated three expression vectors carrying GALC cDNA with "original ATG," "Kozak ATG" and "1st ATG," respectively (Fig. 1). Transient expression studies ($n = 6$) in Bosc23, a cell line derived from the kidney of human embryo, showed no significant difference in GALC expression level among these three constructs. When expression study was performed in NIH/3T3 cells ($n = 3$), expression level from "original ATG" was higher than that of "Kozak ATG" or "1st ATG" with statistically significant difference (Fig. 2). The results indicate that pLHCAG (original) has comparable or better expression level than the other two constructs. And GALC gene with "original ATG" was used in subsequent study.

Epitope tagging of GALC gene

To favor the convenient identification of cells expressing transduced GALC gene in tissues, c-myc-epitope (1 or 3 copies) was introduced to the carboxyl-terminus of GALC with the "original ATG." In a pilot study, immunofluorescence against myc-tag after transfection showed that tandem three copies of the tag significantly improved signal strength comparing with a single copy