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Modulation of lentiviral vector tropism in cerebellar Purkinje cells in vivo by a lysosomal cysteine protease cathepsin K

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Abstract We previously reported that vesicular stomatitis virus-derived glycoprotein (VSV-G)-pseudotyped lentiviral vectors harvested 2 days post-transfection preferred to infect Purkinje cells (PCs), whereas those harvested after a longer cultivation period exhibited Bergmann glia-preferential transduction. However, the mechanisms by which lentiviral tropism was altered remained unsolved. Here, we investigated whether proteases released from the cells during viral production affect lentiviral tropism. Enhanced green fluorescence protein-expressing lentiviral vectors were produced using human embryonic kidney (HEK) 293FT or 293 T cells and injected into the mouse cerebellum to examine tropism in PCs. We found that the addition of a protease inhibitor—in particular, the cathepsin K (CatK) inhibitor—into the culture medium significantly increased lentiviral tropism in PCs. Moreover, the concentration of CatK in the culture medium drastically increased upon prolonged cultivation, concomitant with the expression levels of CatK in HEK 293 T cells. An increase in CatK activity by the addition of recombinant CatK enzyme to PC-preferential viral solution, which was obtained 2 days post-transfection, shifted the viral tropism toward Bergmann glia. In contrast, a decrease in CatK activity in the Bergmann glia-preferential viral solution, which was obtained 6 days post-transfection by the addition of CatK inhibitor or by the removal of a CatK-containing fraction, restored the PC preference of viruses. These results suggest that the CatK released from deteriorated HEK 293 T cells plays a key role in reducing lentiviral tropism in PCs, presumably by affecting a receptor molecule for lentiviral VSV-G, resulting in the preferential transduction of Bergmann glia.

Keywords Lentiviral vector · Purkinje cell · Protease · Cathepsin K · HEK 293

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Introduction

The ability to transfer genes to a specific neuronal population is a promising technique for gene therapy and basic research. The *Lentivirus* genus, which consists of slow viruses of the Retroviridae family, has the potential to insert their proviruses into the genome of post-mitotic and mitotic cells. Vectors derived from lentiviruses are widely used to transfer genes into differentiated neurons. However, the lentivirus from which these vectors were originally derived can only infect CD4-positive lymphocytes because they require the CD4 protein as a receptor. Thus, the lentiviral vectors that are currently used have been engineered with a modified envelope such that the original glycoprotein is replaced with a vesicular stomatitis virus-derived glycoprotein (VSV-G) that binds to membrane phospholipids and confers broad tropism to viral vectors (Yee et al. 1994). This technique is called "pseudotyping," and VSV-G-pseudotyped lentiviral vectors can infect and transduce various types of cerebellar cells such as Purkinje cells (PCs), stellate cells, basket cells, Golgi cells, and Bergmann glia in the cerebellar cortex (Torashima et al. 2006a, b).

Previously, we reported a significant decrease in tropism in PCs by lentiviral vectors produced from prolonged cultivation of human embryonic kidney (HEK) 293FT or 293 T cells (Torashima et al. 2006b). Here, we show that the concentration of cathepsin K (CatK), a lysosomal cysteine protease,

drastically increases in the viral production medium concomitantly with the upregulation of CatK expression in confluent HEK 293 T cells. Moreover, an increase in CatK activity in purified lentiviral solution with the addition of recombinant CatK enzyme significantly reduced viral tropism in PCs. In contrast, a decrease in CatK activity in the viral solution with the addition of a selective CatK inhibitor or the removal of a CatK-containing fraction using centrifugal filter devices significantly increased viral tropism in PCs. These results suggest that CatK released into the medium from degenerated and dead HEK 293 T cells plays a critical role in reducing lentiviral tropism in PCs.

Materials and methods

All procedures for the care and treatment of animals were carried out according to the Japanese Act on the Welfare and Management of Animals and the Guidelines for the Proper Conduct of Animal Experiments issued by the Science Council of Japan. The experimental protocol was approved by the Institutional Committee of Gunma University (11–035 and 07–015).

Virus preparation

Lentiviral vectors expressing enhanced green fluorescence protein (GFP) under the control of the cytomegalovirus (CMV) promoter were produced by transfection of HEK 293FT and HEK 293 T cells with four plasmids, using the calcium phosphate precipitation method as previously described (Torashima et al. 2006b) (Fig. 1). The backbones of the helper plasmid were derived from pCAGGS (Niwa et al. 1991). At 16 h after transfection, the cells were washed twice with phosphate-buffered saline that did not contain calcium or magnesium (PBS (−)), and they were further cultured in fresh medium for one or five more days in the presence or absence of protease inhibitors. The protease inhibitors and their final concentrations in the culture medium were: CGS27023A (Novartis Pharma, Basel, Switzerland), 10 μM; tissue inhibitor of metalloproteinase 1 (TIMP-1) (Abcam, Cambridge, UK), 10 nM; cathepsin inhibitor I (Merck, Darmstadt, Germany), 10 μM; and cathepsin K (CatK) inhibitor (Merck), 10 or 1 μM; the protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan; cat. no. 03969–21) consisted of a mixture of 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) (1 mM), aprotinin (0.3 μM), *N*-[*N*-(L-3-trans-carboxirane-2-carbonyl)-L-leucyl]-agmatine (E-64) (2 μM) and leupeptin hemisulfate monohydrate (2 μM). The medium containing the vector particles was harvested 2 or 6 days after transfection. The media containing the viral particles was filtered through 0.22-μm membranes and centrifuged at 112,708×*g* for 90 min. The viral particles were finally suspended in 70 μl of PBS (−) (pH 7.4) or supernatant (pH approximately 7.0). Viral titers were assessed by counting the GFP-expressing HEK 293 T cells as previously described (Torashima et al. 2006a). In the

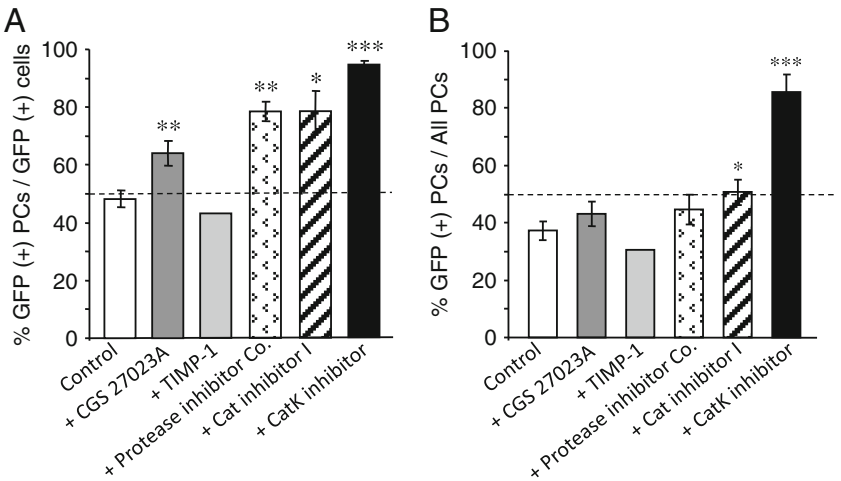

Fig. 1 Significant involvement of a protease in lentiviral tropism in Purkinje cells. **a** The addition of a protease inhibitor to the viral production medium increases the transduction efficiency of lentiviral vectors in Purkinje cells. Lentiviral vectors expressing GFP under the CMV promoter were produced using HEK 293FT cells, fast-growing variants of HEK 293 T cells, in culture medium containing a protease inhibitor. The viral vectors were harvested 2 days after transfection, concentrated by ultracentrifugation, and injected to mouse cerebella. The ratio of GFP (+) PCs to all GFP (+) cells in the cerebellar cortex was determined. **b** The ratio of GFP (+) PCs to total PCs in randomly selected visual fields. The final concentration of protease inhibitors in the culture medium was 10 μM for CGS27023A, 10 nM for TIMP-1, 10 μM for cathepsin inhibitor I, and 10 μM for CatK inhibitor. The protease inhibitor cocktail is a mixture of AEBSF (final concentration, 1 mM), aprotinin (0.3 μM), E-64 (2 μM), and leupeptin hemisulfate monohydrate (2 μM). The *asterisks* indicate statistically significant differences compared with a result obtained using control viral vectors as measured by unpaired *t*-tests; *$p<0.05$, **$p<0.01$, ***$p<0.001$

initial screening, we used HEK 293FT cells, fast-growing variants of HEK 293 T cells, for lentiviral production. The viral titers ranged from 6.3 to 9.7×10^9 transduction units (TU)/ml (n=4–5 batches in each group; mean±S.E.M., $7.4\pm0.3\times10^9$TU/ml). In subsequent experiments, HEK 293 T cells were used to produce lentiviral vectors. Two days after transfection, the average viral titers were $3.7\pm0.9\times10^{10}$TU/ml with a range from 2.5 to 4.9×10^{10} TU/ml (n=4 batches), and 6 days after transfection, the values were $2.2\pm0.4\times10^{10}$TU/ml with a range from 1.5 to 3.3×10^{10}TU/ml (n=6 batches).

Manipulation of CatK activity in the lentiviral solution

CatK activity in the lentiviral solution was increased by directly adding recombinant human CatK enzyme (ENZO Life Sciences, Farmingdale, NY, USA; cat. no. BML-SE553) at a final concentration of 7 μM and 700, 70, or 7 nM. Alternatively, CatK activity in the lentiviral solution was reduced by adding CatK inhibitor (200 nM) or by the removal of a fraction containing CatK (~30 kDa) using a centrifugal filter device (Amicon Ultra-0.5, Ultracel-50 membrane; Millipore, Billerica, MA, USA), which spins down molecules smaller than 50 kDa (50,000 nominal molecular weight limit, NMWL). For CatK spin-down, 500 μl of the lentiviral solution, which was prepared from 10 ml of culture medium 6 days after transfection, was added to the filter device, followed by centrifugation at 14,000×g for 30 min according to the manufacturer's protocol. The lentiviruses remaining in the filter device were recovered by placing the filter device upside down in a clean microcentrifuge tube, and they were spun for 2 min at 1,000×g to transfer the viral particles from the device to the tube. The virus-containing solution for injection was finally adjusted to 70 μl using PBS (−).

Cerebellar injection and quantitative analysis of transduced cell types

Lentiviral vectors were injected into the cerebellar cortex of C57Bl/6 mice following deep anesthesia with sodium pentobarbital (40 mg/g body weight) at post-natal day (P) 21–25 as previously described (Torashima et al. 2006a, b). The mouse was mounted in a stereotactic frame. Then, a midline sagittal incision was made, and the cranium over the cerebellar vermis was exposed. A burr hole was drilled 5 mm caudal to the bregma. The tip of a Hamilton syringe attached to a micropump [UltramicroPump II; World Precision Instruments (WPI), Sarasota, FL, USA] was inserted 0.5 mm from the hole in the dura mater into the molecular layer of the cerebellar vermis (lobule VI). Ten microliters of viral solution was injected at a rate of 200 nl/min using a microprocessor-based controller (Micro4; WPI). This volume and speed were used because, in addition to soaking into the tissue, the viral solution spread over different lobules through the subarachnoidal space, leading to transduction over larger areas (Torashima et al. 2006a). The syringe was left in place for an additional 2 min before it was withdrawn, and the scalp was then sutured. The mouse was maintained on a heating pad until it had recovered from anesthesia and was then returned to a standard cage.

Seven days after the injection, the mice were anesthetized and perfused transcardially with 4 % paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (pH 7.4). The cerebella were isolated and incubated overnight in 4 % PFA at 4 °C and were then cut into 50-μm sagittal sections with a microslicer (DOSAKA DTK-1000, Kyoto, Japan). GFP fluorescence images were obtained using a confocal microscope (LSM 5 PASCAL; Zeiss, Oberkochen, Germany). Three visual fields (0.95 mm^2/field) were randomly selected at×200 magnification from the sagittal sections of the cerebellar vermis. Serial cerebellar sections were scanned at 1-μm thickness and were constructed three-dimensionally. The number of GFP-fluorescent cells in each section was then counted, and PCs and Bergmann glia were identified by their unique morphologies. PCs have a large soma of approximately 20 μm with well-differentiated dendrites, while Bergmann glia have a much smaller soma of ~10 μm, from which a radial process extends to the cerebellar surface. Tropism of lentiviral vectors in PC or Bergmann glia was assessed from the ratio of transduced PCs to transduced Bergmann glia (transduction ratio of PC/BG), which was obtained by dividing the number of transduced PCs by the number of transduced Bergmann glia. Each experiment was performed using more than three mice. The data were analyzed by unpaired t-tests or one-way analysis of variance (ANOVA) followed by Tukey's post hoc test to assess the significant differences, and they are expressed as mean ± S.E.M.

Western blot analysis

HEK 293 T cells were cultured in a 10-cm dish containing 10 ml of medium. The cells were collected and solubilized in 250 μl of lysis buffer consisting of 50 mM PBS (−), 5 mM EDTA, 0.4 % Triton X-100, and a cocktail of protease inhibitors (Nacalai Tesque, Kyoto, Japan: cat. no. 25955–11). The protein concentrations were measured using a non-interfering protein assay kit (NIPA; Millipore). Then, the protein concentration was adjusted to 3 μg/μl, and 20 μl of the sample was mixed with 10 μl of 3× buffer solution containing 250 mM Tris–HCl (pH 6.8), 6 % sodium dodecyl sulfate (SDS), 10 % glycerol, 50 μg/ml bromophenol blue, and 10 % mercaptoethanol.

The CatK concentration in the HEK 293 T culture medium was assessed via western blot analysis 2 and 6 days after

transfection. HEK 293 T cells were cultivated in 10 ml of medium in a 10-cm dish. The culture medium was concentrated using a 10-kDa Amicon Ultra Centrifugal Filter Unit (Millipore; cat. no. UFC801008) according to the manufacturer's protocol. The amount of medium remaining in the upper reservoir was adjusted to 100 μl using PBS (−). Known concentrations of human recombinant CatK enzyme (ENZO Life Sciences; cat. no. BML-SE553) were used as a standard for the assessment of CatK concentration in the culture medium. Ten microliters of 100-fold concentrated medium was mixed with 40 μl of 5× buffer solution and treated as described earlier. The blotted membrane was incubated with mouse monoclonal anti-CatK antibody (BioVendor; 1:5,000), and HRP-conjugated anti-mouse IgG (Bio-Rad; 1:5,000) secondary antibodies were used. Band volume on the western blot was quantified using the Basic Quantifier (Bio Image, Tokyo, Japan).

Regarding VSV-G protein analysis, 1 μl of lentiviral vector solution was diluted with 66 μl PBS (−) and mixed with 33 μl of 3× buffer solution. The solution was incubated at 95 °C for 5 min, and 10 μl of solution was used for immunoblot analysis. Ten percent SDS–polyacrylamide gels was used for sample electrophoresis. The proteins were blotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore) and then blocked with 5 % skim milk in Tris-buffered saline containing 0.2 % Tween-20. The blotted membrane was incubated with a mouse monoclonal anti-CatK antibody (BioVendor, Brno-Modřice, Czech; 1:5,000), mouse monoclonal anti-β tubulin antibody (Sigma, St. Louis, MO, USA; 1:5,000) or rabbit polyclonal anti-VSV-G antibody (Sigma; 1:5,000). The secondary antibodies used were HRP-conjugated anti-mouse and anti-rabbit IgG (Bio-Rad, Tokyo, Japan; 1:5,000). Immunoreactive proteins were visualized with the enhanced chemiluminescence reagent ECL plus (GE Healthcare, Piscataway, NJ, USA).

Quantitative real-time PCR

For the quantitative analysis of CatK mRNA, total RNA from HEK 293 T cells was reverse-transcribed using the PrimeScript II 1st strand cDNA synthesis kit (Takara-Bio, Tokyo, Japan). The measurement was performed using SYBR Premix Ex Taq II (Takara-Bio) on a Thermal Cycler Dice TP800 system (Takara-Bio) with cycles of 95 °C for 5 s and 60 °C for 30 s. The cycle threshold value, which was determined using the second derivative, was used to calculate the normalized expression of the indicated genes using GAPDH as a reference gene. The following primer pairs were used: GAPDH, 5′-ATGGGGAAGGTGAAGGTCG-3′, 5′-GGGGTCATTGATGGCAACAATA-3′; CatK, 5′-CTGCTACCTGTGGTGAGCTTT-3′, 5′-AACGCCGAGAGATTTCATCCA-3′. The results (mean ± S.E.M.) were obtained from three runs of real-time PCR using three different culture samples.

Immunohistochemistry

Mice were perfused transcardially with a fixative containing 4 % formaldehyde in 0.1 M phosphate buffer after being deeply anesthetized with sodium pentobarbital. The whole cerebellum was removed and post-fixed overnight at 4 °C, followed by cryoprotection in 30 % sucrose for 3 h. For immunostaining, the cerebellum was cut into 50-μm sagittal sections using a microslicer (DOSAKA DTK-1000, Kyoto, Japan). Floating sections were immunostained with rabbit polyclonal anti-calbindin D-28 K antibody (1:1,000; Millipore) and mouse monoclonal anti-S100 antibody (1:1,000; Sigma), which were visualized with Alexa fluor-680 Cy3-conjugated anti-goat IgG (Invitrogen, Carlsbad, CA, USA; 1:1,000). Immunofluorescence was evaluated using a confocal laser scanning microscope (LSM 5 Pascal; Zeiss).

N-terminal amino acid sequencing of the VSV-G

Ten microliters of viral solution was subjected to 10 % SDS–polyacrylamide gel electrophoresis. The proteins on the gel were electrophoretically transferred to a PVDF membrane (Immobilon; Millipore) and visualized with Coomassie brilliant blue R-250 (Thermo Scientific, Rockfort, IL, USA). The band corresponding to VSV-G (67 kDa) was excised, washed three times with deionized water, incubated with destaining solution containing 90 % methanol and 7 % acetic acid for 40 s, and applied to a protein sequencer (Outsourced to Nippon Genetics Co. Ltd., Tokyo, Japan).

Results

Protease inhibitors increase lentiviral tropism for PCs in vivo

We first examined whether the tropism of lentiviral vectors in PCs was influenced by the addition of a protease inhibitor into the culture medium during the production of viral vectors. We used two metalloprotease inhibitors (CGS27023A and TIMP-1), one serine/cysteine protease inhibitor cocktail containing AEBSF, aprotinin, E-64 and leupeptin hemisulfate monohydrate, and two cysteine protease inhibitors (cathepsin inhibitor I and CatK inhibitor) selective towards cathepsin, a protease that localizes to the lysosome under physiological conditions. Lentiviral vectors were produced using HEK 293FT cells in the presence of one of the protease inhibitors in the culture

medium. The vectors were harvested 2 days after transfection and injected into the cerebellar cortices of P21–P25 mice. At 7 days after the injection, cerebellar sections of the treated mice were obtained. We determined the ratio of the GFP-expressing PCs to total GFP-expressing cells and the ratio of GFP-expressing PCs to total PCs in three randomly selected visual fields (0.95 mm^2/field) from at least three mice (totaling more than nine visual fields).

When the cerebellum was treated with control lentiviral vectors produced in the absence of protease inhibitors, the number of GFP-expressing PCs was approximately half the total number of GFP-expressing cells (48.1±3.1 %) (Fig. 1a). The ratio significantly increased to 64.1±4.3 % when lentiviral vectors that were produced in the presence of CGS27023A were used; however, lentiviral vectors obtained in the presence of TIMP-1 did not affect the ratio of transduced PCs to total transduced cells (42.3±1.1 %). Notably, the ratio of GFP-expressing PCs increased dramatically to approximately 80 % or higher when we used lentiviral vectors produced in culture media containing cysteine protease inhibitors. The addition of the protease inhibitor cocktail, cathepsin inhibitor I, or CatK inhibitor increased the ratio of GFP-expressing PCs to total GFP-expressing cells to 76.9±3.4, 76.9±7.1, or 94.4±1.5 %, respectively.

Approximately 40 % of PCs of the total number of PCs in a visual field were transduced following the injection of control lentiviral vectors (Fig. 1b). The ratio was not influenced by the addition of CGS27023A, TIMP-1, or the protease inhibitor cocktail; however, it was significantly increased to ~50 or ~90 % by the addition of cathepsin inhibitor I or CatK inhibitor, respectively, to the culture medium. These results suggest that a cysteine protease, specifically CatK, may play a key role in lentiviral vector tropism for PCs.

The expression of CatK significantly increases in HEK 293 T cells after prolonged cultivation

No studies regarding the expression of CatK in HEK 293 cells have been reported. However, a previous study has shown that, although the amount of CatK was negligible in fibroblasts, it increased drastically after the cells became confluent (Quintanilla-Dieck et al. 2009; Runger et al. 2007). Thus, we hypothesized that CatK expression increased in HEK 293 T cells as they reached confluency. Furthermore, we postulated that CatK released from degenerating and dead cells affected the lentiviral vectors, resulting in a decrease in lentiviral tropism in PCs.

To examine whether confluent conditions increase the expression of CatK in HEK 293 T cells, we cultured HEK 293 T cells without viral plasmid transfection until they became confluent. Because HEK 293 T cells grow more slowly than HEK 293FT cells, more than 5 days was needed following plating before the cells became confluent in our culture conditions. Using western blotting and quantitative real-time PCR, we compared the levels of CatK in confluent HEK 293 T cells cultured for 7 days to the levels in sparse HEK 293 T cells cultured for only 3 days. We barely detected a band corresponding to CatK in HEK 293 T cell lysate collected 3 days after plating, whereas a robust CatK band was observed in HEK 293 T cell lysate collected 7 days after plating (Fig. 2a, upper square). We repeated this experiment several times, and in

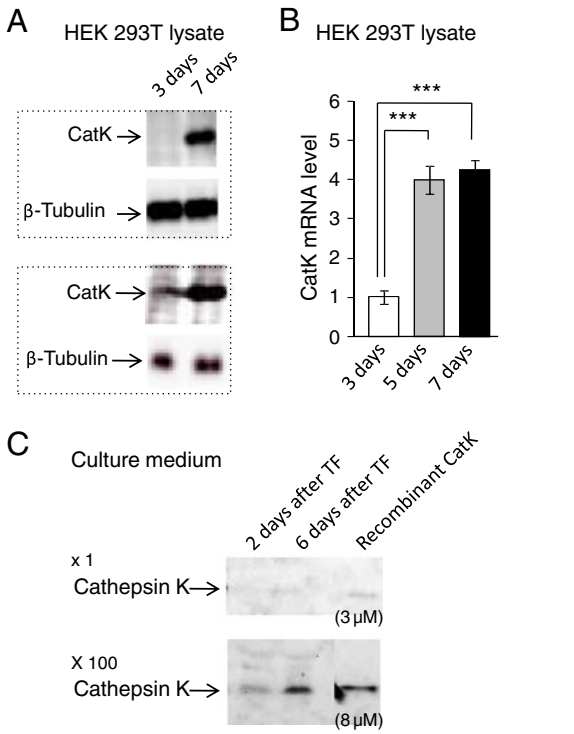

Fig. 2 Western blot analysis of CatK levels in HEK 293 T cells and the culture medium. **a** There is a significant increase in the expression of CatK in HEK 293 T cells after the cells reach confluency. HEK 293 T cells (2×10^6 cells) were cultivated in a 10-cm dish without transfection of any viral plasmids. The cells became confluent 5 days after plating and continued to be cultivated for two more days (total 7 days). The lysates of cells cultured for 3 and 7 days were loaded, and the membrane was immunoblotted for CatK (*upper*) and β-tubulin (*lower*). The results obtained from two independent experiments (*upper* and *lower squares*) are presented. **b** Quantitative RT-PCR analysis of CatK mRNA in HEK 293 T cells cultured for 3, 5, and 7 days. The results were obtained from three runs of real-time PCR using three independent cultures for each group. The *asterisks* indicate statistically significant differences compared with results using a 2-day culture as determined by one-way ANOVA followed by Tukey's post hoc test, ***$p<0.001$. **c** Striking increase in CatK concentration in the culture medium by prolonged cultivation. CatK in the culture medium of HEK 293 T cells 2 and 6 days after transfection was assessed by Western blotting. Recombinant human CatK of known concentrations (3 and 8 μM as indicated) was used as a standard. The upper and lower membranes are the results obtained without (*upper*) and with (*lower*) 100 times concentration of the culture medium

a different trial, an immunoreactive band corresponding to CatK was detected in lysate collected 3 days after plating; however, CatK protein expression levels were much higher in confluent HEK 293 T cells cultivated for 7 days than in exponentially growing cells cultivated for only 3 days (Fig. 2a, lower square). Similarly, by quantitative real-time PCR, the level of CatK mRNA was four times higher in confluent HEK 293 T cells cultured for 5 and 7 days than in sparse HEK 293 T cells cultured for 3 days (Fig. 2b). These results suggest that confluency upregulates CatK expression in HEK 293 T cells as it does in fibroblasts.

Assessment of CatK concentration in the culture medium

To evaluate CatK concentration in the culture medium, the medium cultivating HEK 293 T cells for 2 or 6 days after transfection was subjected to western blot analysis. Human recombinant CatK of known concentrations was used as a standard, and the concentration of CatK in the culture medium was determined by comparing the volume of the bands with that of standard CatK bands. A band immunoreactive to CatK was not detected in culture medium 2 days after transfection; however, a very faint band immunoreactive to CatK was observed in culture medium 6 days after transfection (Fig. 2c, upper image). We then concentrated CatK 100-fold in the culture medium using a centrifugal filter device (10,000 NMWL), and the concentrated medium was subjected to western blot analysis. Clear bands immunoreactive to CatK were observed in both lanes of the culture medium at 2 and 6 days after transfection (Fig. 2c, lower image). Quantification of the band volume in the western blots revealed that the CatK concentration was approximately 75 nM in the culture medium at 2 days after transfection, which drastically increased to approximately 480 nM at 6 days after transfection.

Restoration of lentiviral tropism in PCs by the CatK inhibitor

We produced lentiviral vectors using HEK 293 T cells in the absence or presence of CatK inhibitor (1 μM), and the viral particles were harvested at 2 or 6 days after transfection. The viral vectors were purified and injected into the mouse cerebellum. Figure 3a shows the immunohistochemistry of lentivirus-treated slices double-stained for calbindin, a marker for PCs (blue), and S100, a marker for Bergmann glia (red). PCs have a large soma (~20 μm) with well-differentiated dendrites, while Bergmann glia have a much smaller soma (5~10 μm) with a process extending to the surface of the molecular layer (Fig. 3a). The cell bodies of both PCs and Bergmann glia localize in the PC layer, and they were easily distinguishable from other cortical cells by their unique morphology. The number of GFP-positive cells was not changed significantly by the addition of the CatK inhibitor or the prolongation of the cultivation period (Fig. 3b). The injection of lentiviral vectors harvested 2 days after transfection predominantly transduced PCs with a ratio of 51.5±10.9 % to all transduced cells (transduction ratio of PC/BG=4.9±0.9) (Fig. 3a, d), whereas vectors harvested 6 days after transfection showed a significantly lower transduction ratio in PCs (11.2±0.5 %) but a significantly higher transduction ratio in Bergmann glia (63.3±0.9 %) (transduction ratio of PC/BG=0.2±0.0) (Fig. 3a, d). The addition of CatK inhibitor to the culture medium did not affect the PC preference of viruses harvested at 2 days after transfection (Fig. 3c, d), whereas this treatment significantly suppressed the shift in lentiviral tropism from PCs to Bergmann glia by prolonged cultivation. Furthermore, the injection of lentiviral vectors that were produced in the presence of CatK inhibitor and obtained 6 days after transfection resulted in PC-preferential transduction (transduction ratio of PC/BG=3.5±0.7) (Fig. 3c, d). If lentiviral vectors are modified by CatK during the cultivation period, the addition of CatK inhibitor after the viral harvest should not influence viral tropism in PCs. We tested this hypothesis by applying CatK inhibitor to the lentiviral solution just prior to injection. Lentiviral vectors were harvested 6 days after transfection into HEK 293 T cells. After ultracentrifugation, the precipitates of the lentiviral vectors were resuspended with 70 μl of supernatant, i.e., the culture medium of HEK 293 T cells used for lentiviral production (pH approximately 7.0). The suspension was equally divided into two tubes: one tube received CatK inhibitor at a final concentration of 200 nM just prior to the injection into the cerebellar cortex, and the other tube received control viral solution. Both viral solutions were injected into mouse cerebellar cortices, and the tropism of those vectors in PCs was examined 7 days after injection. The addition of CatK inhibitor to the viral suspension did not alter the number of transduced cells in the cerebellar cortex (Fig. 4a). The control lentiviral vectors had a higher preference for Bergmann glia (48.5±2.2 %) than for PCs (23.2±5.6 %) (transduction ratio of PC/BG=0.5±0.2). In contrast, the lentiviral vectors that were co-injected with CatK inhibitor resulted in a higher transduction ratio for PCs (51.9±5.4 %) than for Bergmann glia (23.6±3.2 %) (transduction ratio of PC/BG=2.3±0.4) (Fig. 4b–d). These results imply that CatK affects cerebellar tissue most likely by regulating a CatK-targeted cell surface molecule to alter lentiviral tropism in PCs, which results in Bergmann glia-preferential transduction.

Bergmann glia-preferential transduction by the addition of recombinant CatK into the lentiviral solution

We used a selective CatK inhibitor, but this compound also inhibits other proteases of the papain family such as

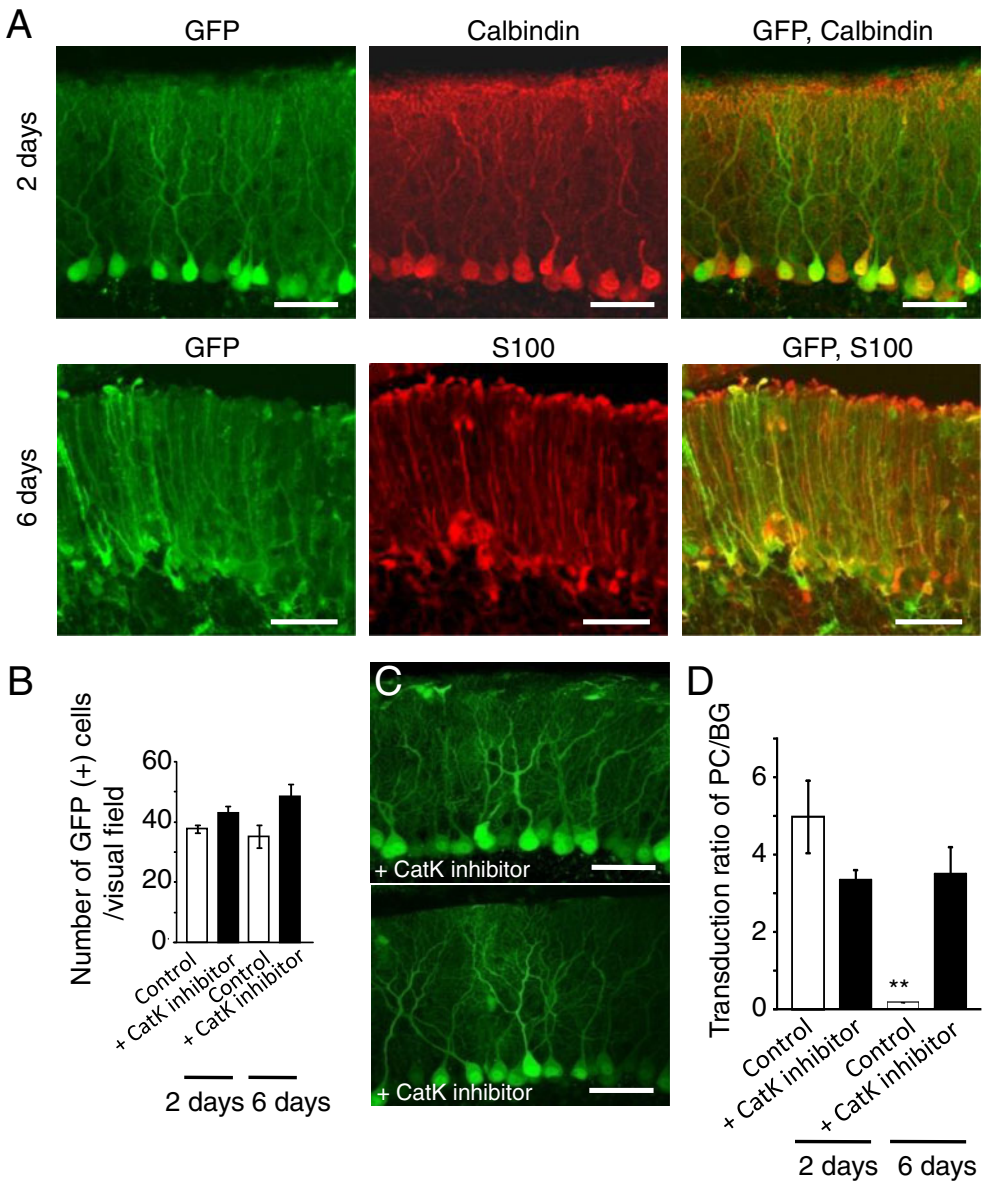

Fig. 3 The prolonged culture-mediated shift of lentiviral vector tropism from PC to Bergmann glia is blocked by the addition of CatK inhibitor to the culture medium. Lentiviral vectors were produced in the absence or presence of 1 μM CatK inhibitor and harvested 2 and 6 days after transfection. The viruses were purified and injected into mouse brain. **a** Transduced cerebellar slices immunolabeled for calbindin, a marker for PCs or S100, a marker for Bergmann glia. The *upper* and *lower panels* are from slices that were treated with lentiviral vectors harvested at 2 and 6 days after transfection, respectively. Merged images of GFP (*left*) and immunofluorescence to calbindin or S100 (*middle*) are shown in the *right panels*. **b** The average number of GFP-expressing cells in one visual field of the cerebellar cortex. Neither the length of culture period nor the addition of CatK inhibitor significantly influenced the potential of the resultant lentiviral vectors in terms of the total number of transduced cells. **c** Representative GFP fluorescence images of cerebellar slices that were treated with lentiviral vectors produced in the presence of the CatK inhibitor and harvested at 2 days (*upper*) or 6 days (*lower*) after transfection. **d** The ratio of transduced PCs to transduced Bergmann glia (transduction ratio of PC/BG). Lentiviral vectors harvested 6 days after transfection showed a significantly reduced transduction ratio of PC/BG, indicating higher tropism in Bergmann glia than in PCs, which was completely counteracted by the use of lentiviral vectors produced in the presence of CatK inhibitor. The *asterisks* indicate statistically significant differences compared with a result obtained using control lentiviral vectors harvested 6 days after transfection as determined by one-way ANOVA followed by Tukey's post hoc test, $^{**}p<0.01$. *Scale bars*, 50 μm

cathepsin L, cathepsin S, and cathepsin B. To confirm the direct involvement of CatK in shifting lentiviral tropism from PCs to Bergmann glia, we added human recombinant CatK enzyme to the solution of PC-preferential lentiviral vectors just prior to injection. The concentration of CatK was adjusted to 7 μM and 700, 70, and 7 nM. The addition of recombinant CatK into the viral solution did not influence the number of transduced cells in the cerebellar cortex

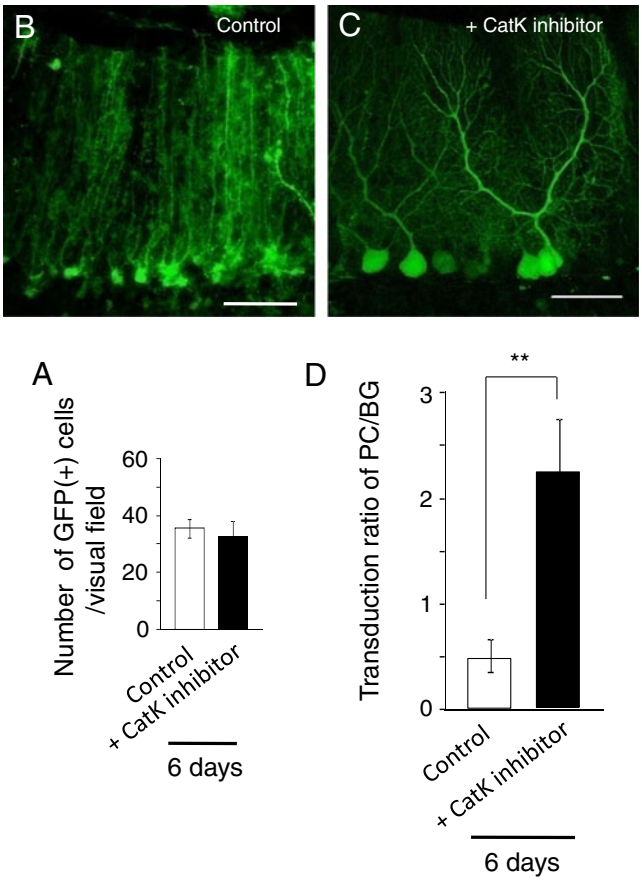

Fig. 4 Restoration of PC-preferential transduction by the addition of CatK inhibitor just prior to brain injection. Lentiviral vectors were produced in the absence of protease inhibitor in the culture medium, harvested 6 days after transfection, and precipitated by ultracentrifugation. The viral particles were then resuspended with the supernatant. Half of the viral solution received the CatK inhibitor (final concentration, 200 nM) just prior to injection into the cerebellar cortex. **a** The average number of GFP-expressing cells in one visual field of the cerebellar cortex. The addition of CatK inhibitor into the viral solution just prior to cerebellar injection did not significantly influence the transduction efficiency. **b**, **c** Representative GFP fluorescence images of sagittal sections of cerebella treated with lentiviral vectors without (**b**) or with (**c**) CatK inhibitor. **d** Quantitative analysis of lentiviral tropism for PCs. The ratio of transduced PCs to transduced Bergmann glia in the cerebellar cortex was examined. Lentiviral vectors harvested 6 days after transfection showed significantly reduced tropism in PCs, which was completely reversed by the addition of CatK inhibitor into the viral solution just prior to the injection into the cerebellar cortex. *Asterisks* indicate statistically significant differences compared with the result obtained using control lentiviral vectors as measured by unpaired *t*-tests; $^{**}p<0.01$. *Scale bars*, 50 μm

(Fig. 5a). Lentiviral vectors that were harvested 2 days after transfection caused PC-preferential transduction (transduction ratio of PC/BG=2.5±0.3). The PC-preference of lentiviral transduction over Bergmann glia was completely reversed by the addition of recombinant CatK at concentrations ranging from 7 μM to 70 nM into the viral solution (Fig. 5c–e, g), whereas the shift of lentiviral tropism from PC to Bergmann glia was not observed when the concentration of recombinant CatK in the viral solution was 7 nM (transduction ratio of PC/BG=1.9±0.1) (Fig. 5f, g).

PC-preferential transduction by removal of a fraction containing CatK from the lentiviral solution

We succeeded in shifting lentiviral tropism from PCs to Bergmann glia by the addition of recombinant CatK enzyme. Next, we tried to reverse the viral tropism from Bergmann glia to PCs by removing CatK from the Bergmann glia-preferential lentiviral solution, i.e., solution obtained 6 days after transfection. CatK has a molecular weight of ~30 kDa and can be spun down using a centrifugal 50 k filter device (50,000 NMWL), while lentiviruses are retained in the upper filter device. After the spin down, lentiviral vectors were recovered, and the volume of lentiviral suspension was finally adjusted to 70 μl with PBS (−). This treatment did not alter the number of transduced cells in the cerebellar cortex (Fig. 6a). Control lentiviral vectors harvested 6 days after transfection caused Bergmann glia-preferential transduction (transduction ratio of PC/BG=0.3±0.0) (Fig. 6b, d). The Bergmann glia-preference of lentiviral transduction over PCs was completely reversed by the removal of the fraction containing CatK (transduction ratio of PC/BG=3.8±0.9) (Fig. 6c, d).

VSV-G is not a target substrate for CatK derived from HEK 293 T cells

We next confirmed that lentiviral vector protein VSV-G was not cleaved by upregulated CatK in the lysosomes of HEK 293 T cells or in the culture medium by western blotting for VSV-G. Lentiviral vectors were produced using HEK 293 T cells and harvested 2 or 6 days after transfection. Using western blot analysis, we observed a single band for VSV-G in HEK 293 T cell lysate obtained 6 days after transfection, and the band size was nearly identical to the size observed 2 days after transfection (data not shown). This result suggests that the lentiviral envelope VSV-G protein was not digested in the culture medium, even 6 days after the transfection. However, if VSV-G was cleaved at a position that was only a few amino acids from the extracellular N-terminal end, it would be difficult to detect a difference in the band size by western blotting. To exclude this possibility, we conducted amino acid sequencing of the extracellular N-terminus of VSV-G. The N-terminal sequence of VSV-G in lentiviral vectors harvested 6 days after transfection was “KFTIV,” which was identical to the sequence in vectors harvested 2 days after transfection. These results indicate that VSV-G is not cleaved by CatK released from HEK 293 T cells, and these results further confirm

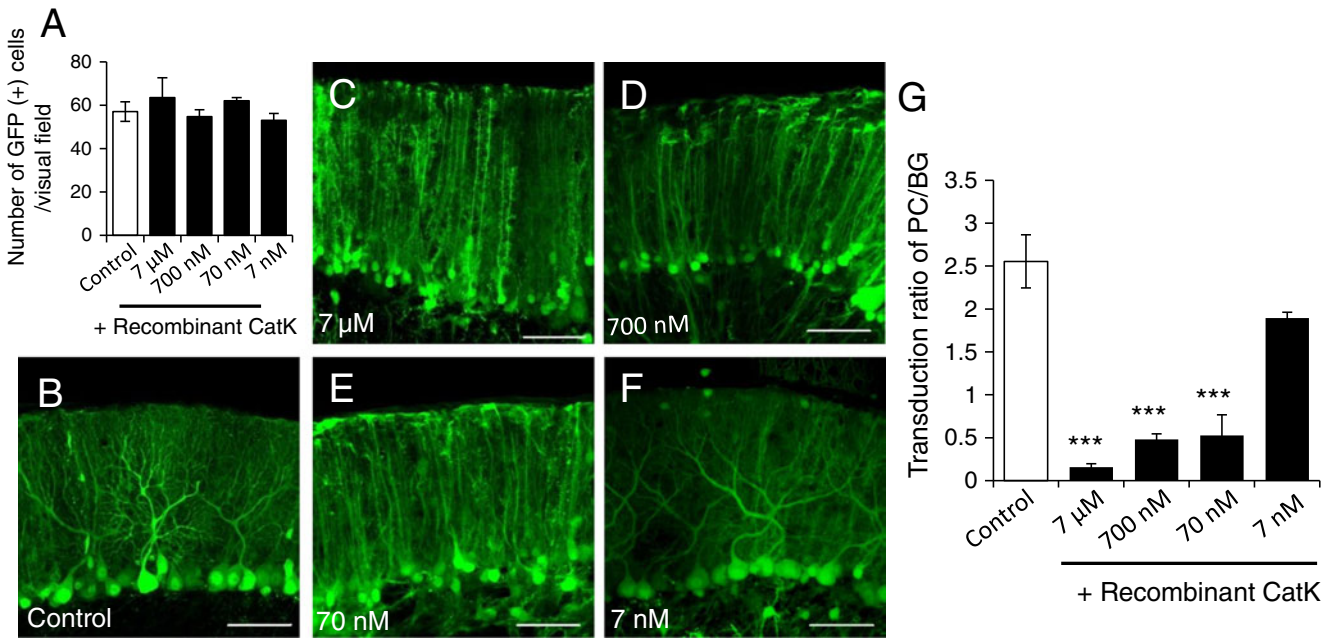

Fig. 5 Drastic shift of lentiviral tropism from PCs to Bergmann glia by the addition of recombinant CatK enzyme to the viral solution. Lentiviral vectors were harvested 2 days after transfection, concentrated by ultracentrifugation, and resuspended with 70 μl of PBS (−) containing recombinant CatK at concentrations of 7 μM and 700, 70, and 7 nM just prior to injection into the cerebellar cortex. **a** The average number of GFP-expressing cells in one visual field of the cerebellar cortex. The addition of the recombinant CatK into the viral solution did not significantly influence the transduction efficiency. **b**–**f** Representative GFP fluorescence images of sagittal sections of cerebella treated with control lentiviral vectors or vectors containing recombinant CatK at the indicated concentration. **g** Quantitative analysis of lentiviral tropism in PCs. The ratio of transduced PCs to transduced Bergmann glia in the cerebellar cortex was examined. Lentiviral vectors harvested 2 days after transfection showed high tropism for PCs, which was clearly shifted toward Bergmann glia by the addition of recombinant CatK at concentrations ranging from 7 μM to 70 nM into the viral solution just prior to the injection into the cerebellar cortex. *Asterisks* indicate statistically significant differences compared with a result obtained using control lentiviral vectors as determined by one-way ANOVA followed by Tukey's post hoc test; ***$p<0.001$. *Scale bars*, 50 μm

that CatK modulates the cortical tissue following injection to alter the lentiviral tropism in PCs.

Discussion

In this study, we demonstrated that the expression of CatK in a low-density culture of HEK 293 T cells drastically increased when the cells reached confluency. Moreover, lentiviral vectors harvested from exponentially growing HEK 293 T cells preferentially transduced PCs in the cerebellar cortex, whereas lentiviral vectors obtained after HEK 293 T cells became confluent were primarily transduced into Bergmann glia. The cell density-dependent change in lentiviral vector tropism from PCs to Bergmann glia was inhibited by the addition of cysteine protease inhibitors, specifically a CatK inhibitor, to the culture medium or to the final viral suspension. Moreover, the ability of the lentiviral solution to preferentially transduce PCs was drastically changed compared to the ability to preferentially transduce Bergmann glia by the addition of recombinant CatK to the viral solution just prior to injection. Alternatively, reduced tropism of lentiviral vectors in PCs was fully restored by the removal of a CatK-containing fraction from the viral solution.

CatK is abundantly expressed in osteoclasts (Bossard et al. 1996; Drake et al. 1996; Garnero et al. 1998); however, recent studies have reported the expression of CatK in dermal fibroblasts (Quintanilla-Dieck et al. 2009; Runger et al. 2007). Consistent with our results obtained using HEK 293 T cells, confluent fibroblasts express much greater amounts of CatK than exponentially growing cells (Quintanilla-Dieck et al. 2009; Runger et al. 2007).

HEK 293 cells were generated by transforming cultures of normal embryonic kidney cells with sheared adenovirus type 5 DNA (Graham et al. 1977). For many years, it was assumed that HEK 293 cells were derived from either fibroblastic, endothelial, or epithelial cells, all of which are abundant in kidney tissue. However, Graham and his coworkers found that HEK 293 cells express the neurofilament subunit α-internexin and many other proteins typically found in neurons, suggesting that the adenovirus transformed a neuronal lineage

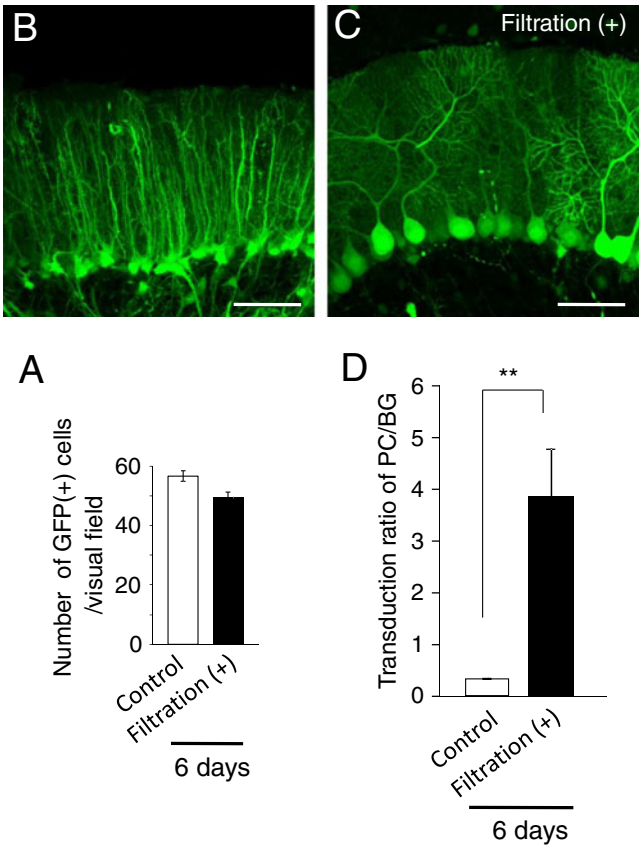

Fig. 6 Significant increase in lentiviral vector tropism in PCs by the removal of a CatK-containing fraction from the viral solution. Lentiviral vectors were harvested 6 days after transfection, precipitated by ultracentrifugation, and resuspended with 500 μl of the supernatant. Molecules less than 50 kDa were removed from the viral solution using a centrifugal filter device. The lentiviral vectors were recovered, resuspended with 70 μl of PBS (−), and injected into the cerebellar cortex. **a** The average number of GFP-expressing cells in one visual field of the cerebellar cortex. Removal of molecules less than 50 kDa from the viral solution did not significantly influence the transduction efficiency. **b**, **c** Representative GFP fluorescence images of cerebellar slices treated with control lentiviral vectors **b** or after the filtration **c**. **d** Quantitative analysis of lentiviral tropism in PCs. The ratio of transduced PCs to transduced Bergmann glia in the cerebellar cortex was examined. Lentiviral vectors harvested 6 days after transfection showed significantly reduced tropism for PCs, which was strikingly increased by the filter-based removal of a fraction containing CatK from the viral solution. *Asterisks* indicate statistically significant differences compared with the result obtained using control lentiviral vectors as measured by unpaired *t*-tests; $^{**}p<0.01$. *Scale bars*, 50 μm

cell present in the original kidney culture (Shaw et al. 2002). It would be intriguing to know whether neurons express CatK. A recent report has shown that CatK is expressed in multiple types of neurons as well as astrocytes, oligodendrocytes, and choroid plexus epithelial cells of the adult human brain (Bernstein et al. 2007). Moreover, there is a clear upregulation of CatK expression in the brain tissue of individuals with schizophrenia compared to matched controls. Interestingly, CatK is abundantly expressed in PCs, and matrix metalloproteinase 9 (MMP9) is expressed in Bergmann glia (Vaillant et al. 1999). During the course of our studies, we found that when the cerebellar tissue was damaged by an injection needle, lentiviral vectors were predominantly transduced into Bergmann glia. This finding may be due to the release of CatK (and maybe, MMP9) from damaged cerebellar tissue, which may modify the cell surface molecules of the surrounding tissue and alter the affinity for lentiviral VSV-G, resulting in the preferential transduction of Bergmann glia.

Amino acid sequencing and western blotting showed that the VSV-G of lentiviral vectors harvested at 6 days after transfection of HEK 293 T cells remained undigested. This finding is consistent with our result that the addition of CatK inhibitor or the removal of a CatK-containing fraction just prior to injection into the cerebellum was sufficient to restore the prolonged culture-dependent decrease in the tropism of lentiviral vectors in PCs (Figs. 4 and 6). If VSV-G is cleaved by CatK during the viral production period, we should not observe the restoration of lentiviral tropism in PCs by decreasing CatK activity after the viral harvest.

If the brain tissue and not the lentiviral particles is the substrate for CatK, CatK should be present in the final lentiviral vector solution. However, in our original virus harvesting protocol, the supernatant from the culture medium after ultracentrifugation was discarded, and the viral precipitate was resuspended with 70 μl of fresh PBS (−). As for this issue, we found that approximately 10–15 μl of the supernatant remained at the bottom of the tube with the viral precipitates, which may have been the source of CatK observed in the final viral solution. Because the viral precipitates in the remaining 10–15 μl of supernatant were resuspended with 70 μl PBS (−), the final concentration of CatK in the viral suspension became approximately one eight of the total in the supernatant. The CatK concentration in the culture medium 2 days after transfection was approximately 75 nM, which increased to approximately 480 nM 6 days after transfection. Then, the final CatK concentrations in the viral solution from cultures 2 and 6 days after transfection were 9 and 60 nM, respectively. The increase in CatK concentration from 9 to 60 nM may be sufficient for the shift of lentiviral tropism from PCs to BG as shown in Fig. 5g.

Lentiviral vector infection is mediated by the interaction of envelope-spiked VSV-G with membrane phospholipids of host cells. Given that the VSV-G is not cleaved by CatK released from HEK 293 T cells, it is conceivable that CatK directly or indirectly modifies the membrane phospholipids of PCs and/or Bergmann glia, resulting in the preferential infection of Bergmann glia in the cerebellum. Further studies are needed to clarify the mechanism regulating the CatK-dependent shift of lentiviral vector tropism from PCs to Bergmann glia.

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