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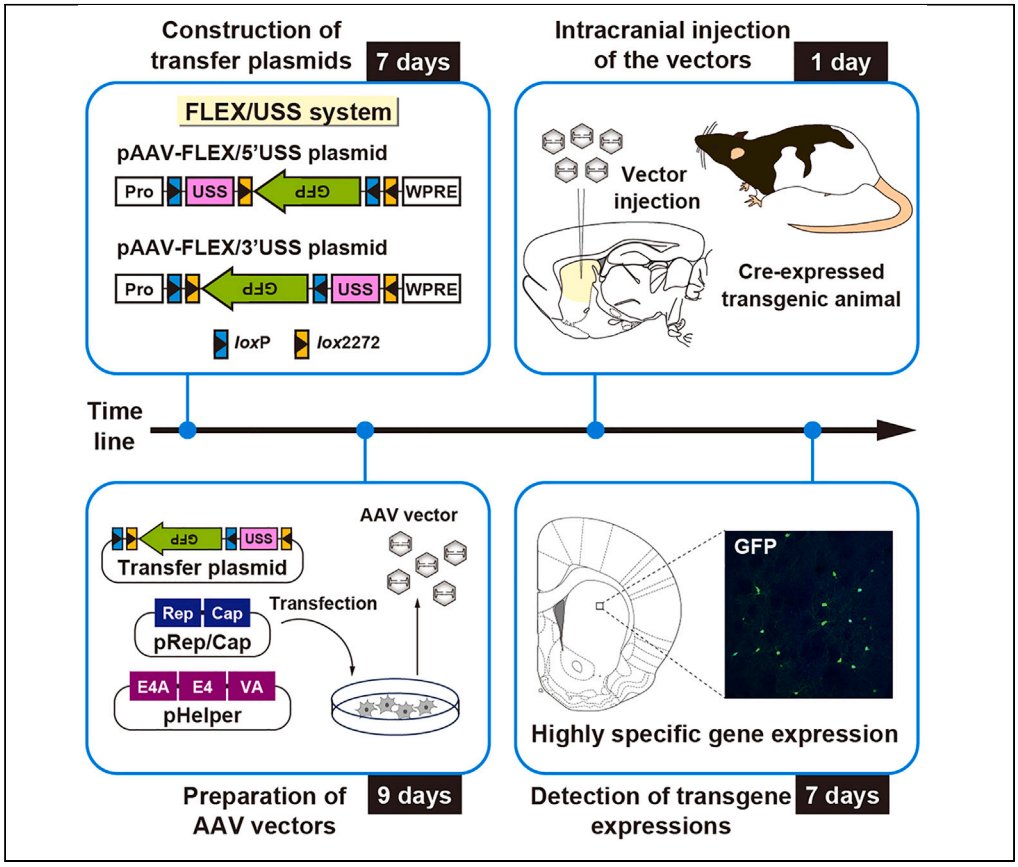
Protocol

Protocol for highly selective transgene expression through the flip-excision switch system by using a unilateral spacer sequence in rodents



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Highlights

Protocol for highly selective Cre-dependent expression of transgene in cell types

Procedures for plasmid construction and AAV vector preparation

Procedures for intracranial injection and transgene detection

Strategy for structural and functional analysis of cell types

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Protocol for highly selective transgene expression through the flip-excision switch system by using a unilateral spacer sequence in rodents

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SUMMARY

We present a protocol to induce Cre-dependent transgene expression in specific cell types in the rat brain, suppressing a leak expression in off-target cells, by using a flip-excision switch system with a unilateral spacer sequence. We describe steps for construction of transfer plasmids, preparation of adeno-associated viral vectors, intracranial injection, and detection of transgene expression. Our protocol provides a useful strategy for a better understanding of the structure and function of specific cell types in the complex neural circuit.

For complete details on the use and execution of this protocol, please refer to Matsushita et al.[1]

BEFORE YOU BEGIN

Cre-*lox*P-mediated recombination is derived from bacteriophage P1 and has been used for the research of a variety of experimental biology. One application of Cre-*lox*P recombination is to express genes of interest in specific cell populations by using the flip-excision switch (FLEX) system[2–4] (see Figure 1A). Adeno-associated viral (AAV) vectors carrying the FLEX structure have been widely used for the purpose of expressing transgenes in specific cell populations in a Cre-dependent manner by using transgenic animals or viral vectors, especially in the neuroscience field.[5–8] However, there is evidence for non-specific expression of transgenes in off-target cell populations after injection of AAV vectors carrying the FLEX system into the tissues.[9] Therefore, a system for more precise and selective control of transgene expression has been needed. Recently, we have developed the FLEX system with a unilateral spacer sequence (USS) in AAV vectors, which suppresses Cre-independent recombination events in viral genome during the vector production in packaging cells, reducing non-specific transgene expression in off-target cells with improved selectivity of gene expression in target cell types expressing Cre recombinase[1] (see Figure 1B). Here, we describe the procedures for construction of transfer plasmids, preparation of AAV vectors, intracranial injection of the vectors, and detection of transgene expression.

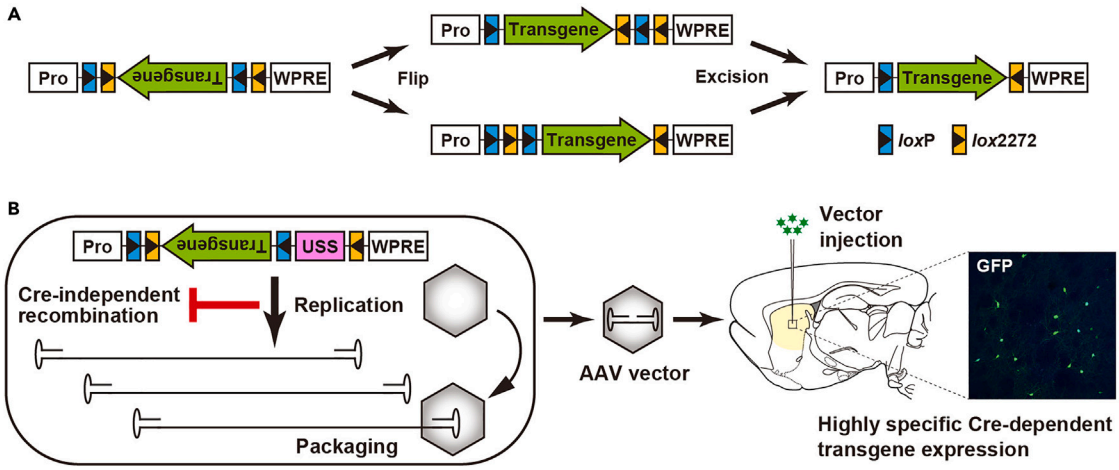

Figure 1. Improvement of the FLEX system by using the USS to suppress Cre-independent recombination

(A) Two-step Cre-dependent recombination of transgene through the FLEX system. In the first reaction, the recombinase catalyzes reversion of the gene cassette through recombination between either of the double recognition sites (flip). In the second reaction, it catalyzes deletion of the intervening sequence between the same two recognition sites (excision) and produces the reversed cassette with different recognition sites at both ends. Pro, promoter.

(B) Improved Cre-dependent transgene expression by using the FLEX/USS system. Introduction of the USS between double recognition sites suppresses Cre-independent recombination events during the production of AAV vector in packaging cells, resulting in enhanced selectivity of transgene expression in target cell types expressing Cre recombinase after the injection of the vector into the brain tissues.

Institutional permissions

Animal care and handling procedures were conducted in accordance with the guidelines established by the Laboratory Animal Research Center of Fukushima Medical University. All procedures were approved by the Fukushima Medical University Institutional Animal Care and Use Committee. The procedures using recombinant *E. coli* or viral vectors must be conducted in the corresponding Biosafety Level (BSL) laboratory with appropriate equipment. The BSL-1 laboratory is required for the preparation of plasmid DNA using *E. coli*, the production of AAV vectors in cultured cells, and other AAV-related experiments. Appropriate disinfection of waste materials is required.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat polyclonal anti-green fluorescent protein (GFP) (1:4000 dilution)	Frontier Science	Cat# GFW-Go-Af1480, RRID:AB_2571574
Rabbit polyclonal anti-parvalbumin (PV) (1:200 dilution)	Frontier Science	Cat# PV-Rb-Af750, RRID:AB_2209751
Rabbit polyclonal anti-red fluorescent protein (RFP) (1:1000 dilution)	Rockland	Cat# 600-401-379, RRID:AB_945213
Mouse monoclonal anti-Cre (1:1000 dilution)	Merck	Cat# MAB3120, RRID:AB_2085748
Donkey anti-goat IgG (H + L) cross-absorbed secondary antibody, Alexa Fluor 488 (1:1000 dilution)	Life technologies	Cat# A-11055, RRID:AB_150129
Donkey anti-mouse IgG (H + L) secondary antibody, Cy3 (1:1000 dilution)	Jackson Immuno Research Laboratories	Cat# 711-165-150, RRID: AB_2307443
Bacterial and virus strains		
DH5α competent cells	Takara Bio Inc.	Cat# 9057
pAAV-FLEX-GFP	Matsushita et al.[1]	Addgene #197883
pAAV-FLEX/5′USS-GFP	Matsushita et al.[1]	Addgene #197884

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
pAAV-FLEX/3′USS-GFP	Matsushita et al.[1]	Addgene #197885
pAAV-FLEX/3′USS(Luc)-GFP	Matsushita et al.[1]	Addgene #197887
pAAV-FLEX/3′USS-ChRWR/Venus	Matsushita et al.[1]	Addgene #197891
pAAV-FLEX/3′USS-hM4Di/2A/GFP	Matsushita et al.[1]	Addgene #197892
pAAV-FLEX/3′USS-mCherry	Matsushita et al.[1]	Addgene #197893
Chemicals, peptides, and recombinant proteins		
Ampicillin sodium salt	SIGMA	A9518-5G
Benzonase	Millipore	71205
Calcium chloride dihydrate	FUJIFILM Wako	039-00475
Cesium chloride (CsCl)	FUJIFILM Wako	036-08165
Disodium hydrogen phosphate 12-water	Nacalai Tesque	31723–35
Dulbecco's modified Eagle's medium (DMEM)	SIGMA	D5796
4′,6-Diamidino-2-phenylindole (DAPI)	Thermo Fisher Scientific	D1306
Ethylenediaminetetraacetic acid, disodium salt, dihydrate (EDTA)	Dojindo	345-01865
Ethanol	FUJIFILM Wako	057-00456
Fetal bovine serum (FBS)	SERENA	29030921
Fluorinert	3M	FC-3283
10% Formalin neutral buffer solution	FUJIFILM Wako	062-01661
Glucose	Fujifilm WAKO	041-00595
HEPES	Dojindo	346-01373
Isoflurane	Pfizer	114-13340-3
Normal swine serum	Vector Laboratories	S-4000
Penicillin-streptomycin solution (×100)	Gibco	15140–122
Potassium chloride	FUJIFILM Wako	163-03545
2-Propanol	Nacalai Tesque	29113–95
Sodium chloride	FUJIFILM Wako	191-01665
Sodium hydroxide	FUJIFILM Wako	198-13765
Sucrose	FUJIFILM Wako	196–00015
Tris(hydroxymethyl)aminomethane	FUJIFILM Wako	512-97505
TritonX-100	FUJIFILM Wako	807426
TrypLE select enzyme (10×)	Gibco	A1217701
Critical commercial assays		
AAV Helper-Free System	Agilent Technologies	Cat# 240071
Gibson Assembly Cloning Kit	New England Biolabs	Cat# E5510S
TaqMan Universal PCR Master Mix	Thermo Fisher Scientific	Cat# 4304437
NucleoBond Xtra Maxi EF Kit	Macherey-Nagel	Cat# 740426
Experimental models: Cell lines		
HEK293T cell line	American Type Culture Collection	Cat# CRL-11268
Experimental models: Organisms/strains		
Rat: Long Evans, 12-week-old male	Jackson Laboratories Japan	N/A
Rat: PV-Cre knockin, 12-week-old male	NBRP-rat in Japan	N/A
Oligonucleotides		
PCR primers	Matsushita et al.[1]	See primer list table
Other		
Cell scraper	FALCON	353085
Cell culture dish (100 mm)	Violama	2-8590-03
0.45-μm filter	Kurabo	S-2504
22-gauge needle	Terumo	NN-2225R
25-gauge needle	Terumo	NN-2525R
Glass capillary	Sutter Instruments	B100-75-10
Hamilton syringe	HAMILTON	84875_1801N
OCT compound	Sakura	4583

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Pasteur pipette	Iwaki	11-063-004
Quick-seal tube	Beckman Coulter	342413
Thinwall polypropylene tube	Beckman Coulter	326819
Slide-A-Lyzer G2 dialysis cassette (10 kDa MWCO)	Thermo Fisher Scientific	87730
1.5-mL tube	BIO-BIK	CF-0150
15-mL tube	FALCON	352096
50-mL tube	FALCON	352070
Vivaspin Turbo 4 (10 or 100kDa MWCO)	Sartorius	VS.04T02, VS.04T42
Anesthesia equipment	Natsume	KN-1071
Constant temperature water bath	YAMATO	BF400
Microinfusion pump	EICOM	ESP-32
Micropipette puller	NARISHIGE	PC-10
Peristaltic pump	Thermo Fisher Scientific	7528-10
Stereotaxic manipulator	NARISHIGE	SR-5R-HT
Surgical microscope	Leica	M320TC12
Ultracentrifuge equipment	Beckman Coulter	Optima L-100K
Ultracentrifuge near-vertical tube rotor	Beckman Coulter	NVT 65
Ultracentrifuge swinging-bucket rotor	Beckman Coulter	SW 55Ti

MATERIALS AND EQUIPMENT

HEK293T cell culture medium

Reagent	Final concentration	Amount
DMEM	NA	445 mL
FBS	10%	50 mL
Penicillin-streptomycin solution (×100)	×1	5 mL
Total		500 mL

Trypsin-EDTA solution

Reagent	Final concentration	Amount
TrypLE select enzyme (10×)	1×	5 mL
0.5 M EDTA	1 mM	0.1 mL
PBS	N/A	44.9 mL
Total		50 mL

10× HBSP stock solution

Reagent	Final concentration	Amount
Sodium chloride	1.4 M	40.9 g
HEPES	50 mM	29.8 g
Disodium hydrogen phosphate 12-water	7.5 mM	1.35 g
Potassium chloride	50 mM	1.9 g
Glucose	60 mM	5.4 g
ddH_2O	N/A	>400 mL
Total		500 mL

Note: 10× HBSP stock solution should be filtered to sterile through a 0.45-μm filter.

2× HBSP solution		
Reagent	Final concentration	Amount
10× HBSS	2×	100 mL
1 M Sodium hydroxide	Adjust to pH 7.05–7.20	N/A
ddH_2O	N/A	> 300 mL
Total		500 mL

Note: 2× HBSP solution should be filtered to sterile through a 0.45-μm filter, and divided into 50-mL aliquots.

Calcium chloride solution		
Reagent	Final concentration	Amount
Calcium chloride dihydrate	2.5 M	14.7 g
ddH_2O	N/A	> 30 mL
Total		40 mL

Note: Calcium chloride solution should be prepared on ice, filtered to sterile through a 0.45-μm filter.

Tris-buffered saline (TBS)		
Reagent	Final concentration	Amount
Tris(hydroxymethyl)aminomethane	5 mM	6.1 g
Sodium chloride	13.8 mM	8.1 g
Potassium chloride	0.27 mM	0.2 g
ddH_2O	N/A	>900 mL
Total		1 L

0.1 M Phosphate buffer (PB)		
Reagent	Final concentration	Amount
Disodium hydrogen phosphate 12-hydrate	0.094 M	33.7 g
Sodium dihydrogen phosphate dihydrate	0.021 M	3.31 g
ddH_2O	N/A	> 900 mL
Total		1 L

Mixed anesthetic solution		
Reagent	Final concentration	Amount
Medetomidine hydrochloride (1 mg/mL)	30 μg/mL	0.75 mL
Midazolam (5 mg/mL)	400 μg/mL	2.0 mL
Butorphanol tartrate (5 mg/mL)	500 μg/mL	2.5 mL
5 M Sodium chloride	118 mM	0.59 mL
ddH_2O	N/A	19.1 mL
Total		25 mL

STEP-BY-STEP METHOD DETAILS

Construction of transfer plasmids

Timing: 7 days

In this section, we describe how to construct the transfer plasmids for preparation of AAV vectors.

1. Prepare the plasmid containing a multicloning site (MCS) between the cytomegalovirus early enhancer/chicken β-actin (CAG) promoter and woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) sequence connecting to human growth hormone polyadenylation signal, resulting in pAAV-CAG-MCS-WPRE. Place a pair of *lox*P-*lox*2272 sequences into the MCS of the plasmid in the opposite direction with an alternate order by using synthetic DNA.
2. Construct the transfer plasmid termed pAAV-CAG-FLEX-GFP by inserting GFP cDNA in the inverted orientation between a pair of the double recognition site sequences in pAAV-CAG-MCS-WPRE by using a Gibson Assembly Cloning Kit (New England Biolabs).
   a. If necessary, replace the CAG promoter with another suitable promoter, such as the human elongation factor-1α (EF1α) promoter.
   b. For other transfer plasmids, replace the GFP cDNA with other genes of interest, such as red fluorescent protein (RFP; mCherry), a variant of channelrhodopsin-2 fused to Venus (ChRWR/Venus), or a mutant form of human muscarinic M4 acetylcholine receptor connected to 2A-GFP (hM4Di/2A/GFP).
3. Cleave the pAAV-CAG-FLEX-GFP plasmid with *Eco*RI or *Cla*I at either side of the inverted transgene and insert the USS of approximately 750 bp using Gibson Assembly Cloning Kit. For instance, the gene cassette encoding TurboFP635, a mutant of TurboFP635 (mTurboFP635) or marine copepod luciferase (Luc) are inserted between the *lox*P and *lox*2272 sites either at the 5′- or 3′-end of the transgene, resulting in pAAV-CAG-FLEX/5′USS-GFP or pAAV-CAG-FLEX/3′USS-GFP.
4. Transform *E.coli* DH5α strain with the respective plasmids, pick up colonies, culture each colony in 3 mL of LB medium containing 50 μg/mL of ampicillin at 37°C for 8 h, and then scale up to 500 mL to be cultured for 16 h.
5. Prepare the plasmids using the NucleoBond Xtra Maxi EF Kit (Macherey-Nagel).

Note: 108 μg of transfection grade plasmid is necessary for transfecting eighteen 100-mm dishes of HEK293T cells. The pAAV-CAG-FLEX-GFP, pAAV-CAG-FLEX/5′USS-GFP, and pAAV-CAG-FLEX/3′USS-GFP can be obtained from Addgene (#197883, #197884, and #197885, respectively).

Pause point: Plasmids can be stored at −20°C or −80°C for years.

Preparation of AAV vectors

Timing: 9 days

In this section, we describe how to produce AAV serotype 2 vectors (AAV-CAG-FLEX-GFP and AAV-CAG-FLEX/3′USS-GFP) based on AAV Helper-Free System (Agilent Technologies) by transfecting the plasmids into HEK293T cells with the calcium phosphate co-precipitation method. Transfection into the cells is also carried-out with polyethylenimine or lipofectamine. The vectors are purified by using the CsCl gradient ultracentrifugation. Purification processes are summarized in Figure 2. The procedures for preparation of AAV vectors are also described in some previous reports.[5,10,11]

Required amount of plasmid DNA

Reagent	Final concentration	Amount
AAV transfer plasmid (pAAV-CAG-FLEX-GFP or pAAV-CAG- FLEX/3′USS-GFP)	N/A	108 μg
Replication/encapsulation gene plasmid (pRep/Cap)	N/A	108 μg
Adeno-helper gene plasmid (pHelper)	N/A	108 μg

Note: 6 μg of each plasmid is required for transfection per a 100-mm dish. In the case of a 150-mm dish, 16.8 μg of each plasmid is required.

DNA-HBSP transfection mix		
Reagent	Final concentration	Amount
Three types of plasmids	6 μg/mL each	108 μg each
2.5 M Calcium chloride solution	125 mM	0.9 mL
2× HBSP solution	1×	9 mL
ddH_2O	N/A	8.1 mL
Total		18 mL

Note: A total of 18 mL of the transfection mix solution is used for eighteen 100-mm dishes.

6. Culture HEK293T cells in 100-mm cell culture dishes containing 7 mL of DMEM supplemented with 10% FBS and penicillin-streptomycin in a 37°C, 5% CO_2 humidified incubator.

Note: The cell culture medium should be stored at 4°C, and pre-warmed to 37°C before use.

The cells should be subcultured before becoming confluent on the plate.

7. Maintain the cells until approximately 80% confluent.
8. Aspirate medium, wash cells once with 5 mL of PBS, and remove residual medium in the plate.
9. Detach cells with 2 mL of Trypsin-EDTA solution for 3 min at 37°C.
10. Neutralize the enzymatic activity of trypsin by adding 10 mL of pre-warmed culture medium and suspend the cells by gentle pipetting.
11. Transfer the suspended cells to 50-mL tubes.
12. Centrifuge the tubes at 300 × *g* for 5 min at 4°C and discard the supernatant.
13. Resuspend the cell pellet in an appropriate volume of pre-warmed cell culture medium.
14. Add the suspended cells to six 100-mm cell culture dishes containing 7 mL of cell culture medium (approximately 1 mL to each dish).

Note: After dropping the suspended cells into each dish, shake the dish gently to spread the cells over culture medium.

15. Incubate the cells for 24 h in a 37°C, 5% CO_2-humidified incubator.
16. Prepare a transfection mixture to transfect the plasmids into the cells in eighteen 100-mm culture dishes.
    a. Mix 108 μg for each of AAV transfer (pAAV-CAG-FLEX-GFP or pAAV-CAG-FLEX/3′USS-GFP), pRep/Cap, and pHelper plasmids (2 mg/mL for each plasmid) in 8.1 mL of ddH_2O and 0.9 mL of 2.5 M calcium chloride solution in a 50-mL tube.
    b. Add 9 mL of 2× HBSP solution dropwise, mixing gently with the vortex mixer.
    c. Incubate the mixture for 5 min to form the DNA-HBSP complex.

Note: The 10× HBSP stock solution should be stored at 4°C, and the 2× HBSP solution should be stored at −20°C. After thawing, the solution should be stored at 4°C. The 2.5M Calcium chloride solution should be stored at −20°C.

17. Drop 1 mL of the mixture into each 100-mm culture dish containing 7 mL of culture medium, and shake the dish gently.
18. Incubate the cells for 18 h in a 37°C, 5% CO_2 humidified incubator.
19. Aspirate the medium, add 7 mL of the pre-warmed fresh culture medium, and incubate the cells for 2 more days.
20. Remove the medium, add 2 mL of PBS per dish, strip the cells with a cell scraper, and collect them into a 50-mL tube.
21. Centrifuge at 300 × *g* for 5 min at 4°C and remove the supernatant.
22. Add 10 mL of PBS, wash cells, and remove supernatant by centrifugation (300 × *g*) for 5 min at 4°C.

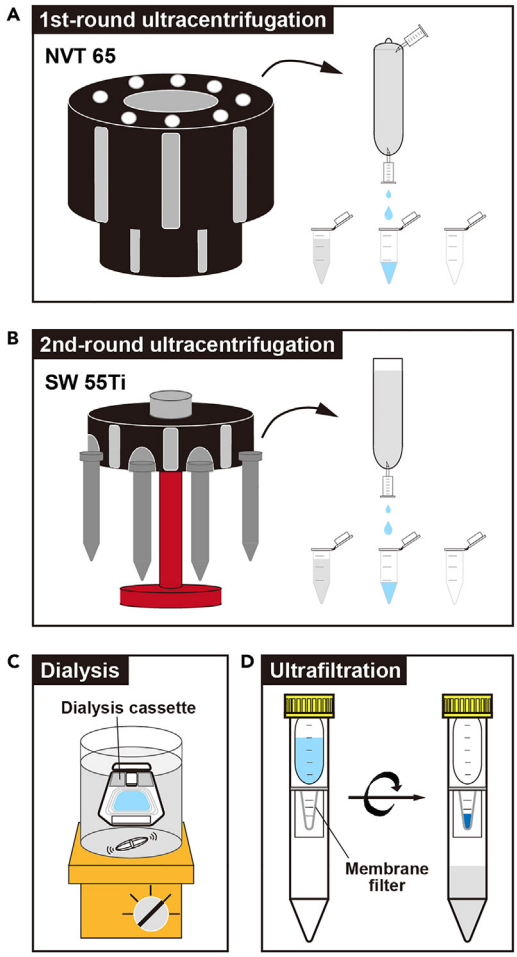

Figure 2. Purification processes of AAV vector particles

The crude viral vector lysate, which is prepared from the cultured cell medium after DNA transfection, is purified with the first round of CsCl gradient ultracentrifugation by using a near-vertical tube rotor (NVT 65) with quick-seal tubes (A), and then the second round of CsCl gradient ultracentrifugation by using a swinging-bucket rotor (SW 55Ti) with thinwall polypropylene tubes (B). The solution containing the particles is collected as drops through the needle inserted into the bottom of the tubes. The solution is dialyzed against PBS by using a dialysis cassette (C), and vector particles are concentrated by ultrafiltration through a membrane filter (D).

Pause point: The resultant cell pellet can be stored at −80°C.

23. Resuspend the cell pellet in 6.5 mL of TBS.
24. For cell lysis, freeze the pellet in liquid N_2 for 5–10 min and thaw in a 37°C water bath for 5–10 min. Shake occasionally the suspension to lysate the cells and release vector particles.
25. Repeat the freeze-thaw cycle three times.

Pause point: Frozen cell suspensions prior to the third thaw can be stored at −80°C.

26. Add benzonase into the tube to a final concentration of 50 U/mL and mix gently the solution.
27. Place the tube in a water bath at 37°C for 30 min to degrade DNA in the cell lysate.
28. Add 65 μL of 0.5 M EDTA to stop the reaction.
29. Centrifuge at 3,500 × *g* for 15 min at 4°C.

30. Transfer the supernatant containing vector particles to a 15-mL tube. The volume of the supernatant is approximately 5–7 mL.

Note: Store the supernatant at 4°C during the preparation of the CsCl gradient.

31. Fill up the supernatant to 8.11 mL with PBS, and add 4.59 mL of saturated CsCl/PBS solution (1.24 g/mL) (total volume 12.7 mL).
32. Transfer the AAV vector solution to a quick-seal tube using a Pasteur pipette.
33. Add saturated CsCl/PBS solution into the tube until the surface reaches the tube's hole neck, and then check the balance.
34. Seal the tube top using a heat-sealer.
35. Centrifuge in a Beckman NVT 65 rotor at 287,000 × *g* (55,000 rpm) for 24 h at 16°C.

Note: After ultracentrifugation, carefully carry the rotor to avoid collapsing the gradient.

1st-round ultracentrifugation

Reagent	Amount
Crude AAV vector solution (supernatant)	5–7 mL
PBS	To make 8.11 mL
CsCl/PBS solution (1.24 g/mL)	4.59 mL
Total	12.7 mL

36. Insert a 22-gauge needle into the top of the tube for venting, and another 22-gauge needle into the bottom of the tube.
37. Collect approximately 1.0 mL of drop solution from the bottom into a 1.5 mL tube (1–10 fractions).
38. Pool the fractions containing vector particles to a thinwall polypropylene tube and balance with CsCl/PBS solution (0.55 g/mL).

2nd-round ultracentrifugation

Reagent	Amount
Collected AAV vector solution	5 mL
CsCl/PBS solution (0.55 g/mL)	For balance
Total	5 mL

Note: In our protocol, the second to sixth fractions usually contain vector particles. Real-time quantitative PCR can be performed using each fraction as a template to confirm the peak fraction.

39. Centrifuge in a Beckman SW 55Ti rotor at 287,000 × *g* (48,700 rpm) for 24 h at 16°C.

Note: After ultracentrifugation, carefully carry the rotor to avoid collapsing the gradient.

40. Pierce the bottom of the tube with a 22-gauge needle to collect 0.5 mL of drop solution from the bottom into a 1.5 mL tube (1–10 fractions).
41. Take 1 μL of solution from each fraction, dilute 1000-fold with ddH_2O, and use 4 μL of each dilution for the quantitative PCR as a template.
42. Dialyze the collected vector solution with a Slide-A-Lyzer G2 dialysis cassette in 1 L of PBS at 4°C twice for 2 h.

43. Dialyze the vector solution once again for 12–16 h at 4°C in 1 L PBS.
44. Wet the membrane filter of Vivaspin Turbo 4 by adding 2–3 mL of PBS, centrifuge at 3,500 × *g* for 1 min at 4°C, and discard the flow-through PBS.
45. Centrifuge the vector solution using the Vivaspin Turbo 4 at 3,500 × *g* for 30–45 min at 4°C until the volume reaches about 100 μL.

Note: Usually, the 4th to 7th fractions are collected after the vector titration.

46. Collect the solution into a 1.5-mL tube.
47. Centrifuge at 13,000 × *g* for 20 min at 4°C to remove any protein residues.
48. Collect the supernatant solution (approximately 50–100 μL) containing AAV particles to a 1.5-mL tube.
49. The vector solution is aliquoted into 5–10 μL and stored at −80°C for at least 1 year.

Note: Take the solution of 2 μL for vector titration.

50. Prepare a standard curve with AAV transfer plasmid (pAAV-CAG-FLEX-GFP or pAAV-CAG-FLEX/3′USS-GFP). Make serial dilutions of the standard plasmid in 10-fold dilutions from 1 × 10^{10} to 1 × 10^{7} copies/mL.
51. Dilute the vector solution at 1:100 and 1:1,000 with ddH_2O.
52. Prepare the reaction mixture with TaqMan Master Mix.
53. Perform the real-time quantitative PCR and determine the vector titer based on the standard curve. See troubleshooting 1 and 2.

PCR primers

Name	Target	Sequence	Source
Fw-W	WPRE	5′ CCGTTGTCAGGCAACGTG 3′	Thermo Fisher Scientific
Rv-W1	WPRE	5′ AGCTGACAGGTGGTGGCAAT 3′	Thermo Fisher Scientific
TM-W	WPRE	5′-FAM TGCTGACGCAACCCCCACTGGT MGB-3′	Thermo Fisher Scientific

PCR reaction master mix

Reagent	Final concentration	Amount
2× TaqMan Universal PCR Master Mix	1×	10 μL
100 μM Forward primer	900 nM	0.18 μL
100 μM Reverse primer	900 nM	0.18 μL
10 μM TaqMan probe	250 nM	0.5 μL
PCR template (AAV samples or standard plasmids)	N/A	4 μL
ddH_2O	N/A	5.14 μL
Total		20 μL

PCR cycling condition

Step	Temperature	Time	Cycle
Initial denaturation	95°C	10 min	1
Denaturation	95°C	15 s	40
Annealing and extension	60°C	60 s	40
Hold	4°C	∞	

Note: Perform the melt curve analysis, in which the temperature of PCR products is shifted from 60°C to 95°C with the increment of 0.5°C /min, to confirm the specificity of DNA amplification during the quantitative PCR.

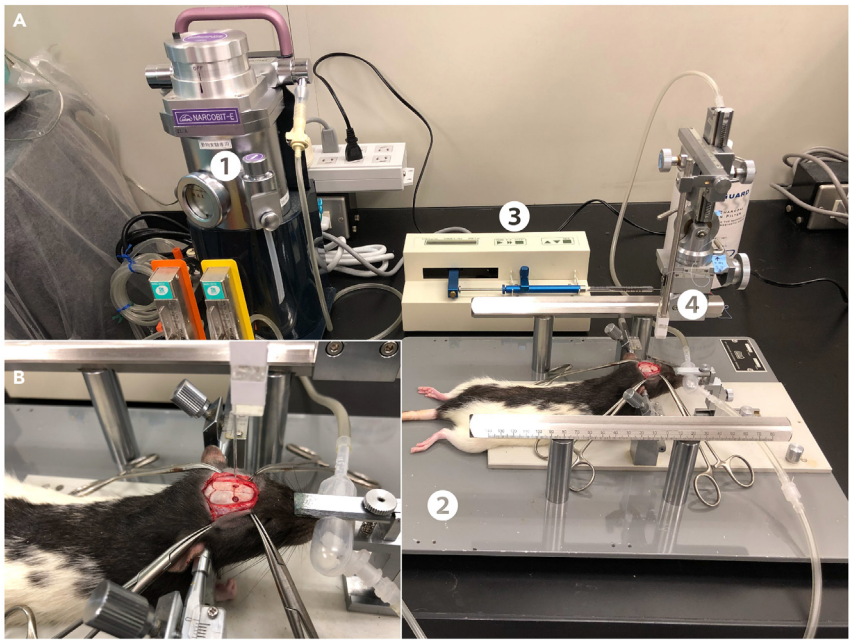

Figure 3. Intracranial injection of AAV vectors

(A) Overall view of AAV vector injection into the rat brain. A deeply anesthetized rat is placed in a stereotaxic instrument to inject AAV vector solution into the brain region. 1) isoflurane anesthesia apparatus; 2) stereotaxic instrument; 3) microinfusion pump, and 4) micropipette manipulator.

(B) Injection of AAV vector solution is performed by inserting the glass micropipette into the brain region according to the coordinates from bregma and dura.

Intracranial injection of the vectors

Timing: 1 day

Here we describe how to prepare the surgical supplies, stereotaxic instruments, and glass micropipettes, and mention the method for stereotactic injection of AAV vector solution into the striatum of the wild-type (Long Evans) and parvalbumin (PV)-Cre knockin rats, in which Cre recombinase is expressed under the control of the PV gene promoter. AAV vectors were introduced into the striatum (4 sites), and the anteroposterior (AP), mediolateral (ML), and dorsoventral (DV) coordinates (mm) from bregma and dura were 1.5/3.0/3.0 (site 1), 1.5/3.0/3.5 (site 2), 0.5/3.0/3.0 (site 3), and 0.5/3.0/3.5 (site 4) according to an atlas of the rat brain.[12] A set of stereotaxic injection into the rat brain is shown in Figure 3.

54. Sterilize surgical instruments and supplies such as forceps, fine scissors, and swabs by using an autoclave.
55. Sterilize the surgical area with a sterilizing disinfectant.
56. Prepare the glass micropipettes (10 cm in length, 1.0 mm in outer diameter, 0.75 mm in inner diameter) for vector injection.
    a. Pull borosilicate glass capillary using a micropipette puller (Model PC-10, NARISHIGE). The parameter of a pulling program is: Step1, Level at 65.
    b. Cut the pipette tip with scissors (outer diameter of the tip: 12–14 μm).
57. Apply the anesthesia to a rat and fix the head in a stereotaxic instrument.
    a. Anesthetize the rat intraperitoneally (i.p.) with the mixed anesthetic solution including medetomidine (0.04 mg/kg), midazolam (0.4 mg/kg) and butorphanol (0.4 mg/kg).
    b. Place the rat in a sealed anesthesia induction chamber and supply 4% isoflurane for 3–5 min.

Note: The anesthetic solution should be stored at 4°C within 8 weeks.

c. Shave the head with an electric clipper/trimmer, being careful not to wake up from the anesthesia.
d. Fix the head to the stereotaxic frame with two ear bars and a nose clamp.
e. Attach the anesthesia head holder and supply 1.5% isoflurane.
f. Confirm that the rat is in deep anesthesia by monitoring the toe reflex. If there is no reflex, isoflurane concentration can be lowered to 1%.
g. Monitor deep anesthesia with periodic toe-pinches during surgery.
h. Apply eye lubricant to protect eyes from drying.

58. Determine the drilling points in the rat brain.
    a. Wipe the head with 70% ethanol to remove any hair left on the skin after shaving.
    b. Disinfect the scalp with 10% povidone-iodine at least three times.
    c. Incise the scalp with thin scissors (approximately 20-mm length).
    d. Clean the skull with a cotton swab dipped in sterile PBS so that bregma and lambda are clearly visible.

Note: Bregma is the intersection of the coronal and sagittal sutures of the skull, and lambda is the intersection of the lambdoid and sagittal sutures. For accurate anatomical positioning of the brain, both the anteroposterior (AP) and mediolateral (ML) axes of the skull must be horizontal to the plate of the stereotaxic instrument.

e. Adjust the tilt of the head so that bregma and lambda are in the same horizontal plane.

Note: The difference between the dorsoventral (DV) coordinates of bregma and lambda should be less than 0.05 mm.

f. Set the coordinates of the bregma as the origin of the coordinates.
g. Connect the 0.5-mm round stereotaxic drill to the stereotaxic manipulator.
h. Rotate the AP and ML modulators and position them so that the drill tip moves to the coordinates over the left or right striatum.
i. Rotate slowly the DV modulator under the surgical microscope and lower the drill until the tip of the drill reaches the skull surface.
j. Drill into the skull carefully and slowly while looking through the surgical microscope.

Note: Drill through the targeted skull point until the dura mater, which lies beneath the skull, is visible. The dura mater is often punctured during drilling. If not, the mater is ruptured with a 25-gauge needle tip. Be careful not to damage the brain parenchyma.

⚠ CRITICAL: Bleeding may occur when puncturing the skull. In this case, stop the bleeding by pressing the hole with a small cotton swab dipped in sterile, cold PBS. Bone debris and excess blood should be washed away. Before injecting the vectors, cover the hole with a sterile PBS-soaked cotton to moisten the surface of the brain.

59. Inject AAV vector solution into the brain regions.
    a. Fill the glass micropipette with Fluorinert (Sumitomo 3M), and connect to the Hamilton syringe through the teflon tube.
    b. Thaw the AAV vector solution on ice.

⚠ CRITICAL: Avoid multiple freeze-thaw cycles of AAV solution to prevent a loss of transduction efficiency of vectors. If the same vector solution is used within a week, store it at 4°C.

c. Pull the plunger of the Hamilton syringe to draw up the vector solution into the glass micropipette.
d. Set the glass micropipette to a stereotaxic manipulator.
e. Move the glass micropipette over the hole in the left or right striatum and slowly rotate the DV modulator to lower the tip so that the tip is close to the surface of the brain, and set the surface as a reference point for the DV coordinate.
f. Penetrate the brain by lowering the tip of the glass micropipette until it reaches the depth of the striatum from the surface.

Note: Once the tip of the glass micropipette reaches the striatum, leave it for 1–2 min.

g. Inject 500 nL of vector solution into the striatum at a flow rate of 50–100 nL/min.
h. Placed the glass micropipette in the end position to spread the vector for an additional 5 min to prevent the solution backflow.
i. Retract slowly the glass micropipette until the tip is completely away from the skull.
j. Repeat as above for other striatal regions.
k. Close the wound with medical surgical sutures.
l. Apply 0.1% GENTACIN Ointement to the suture to prevent surgical would infection.
m. Return the rat to the home cage.

Note: It is better to keep the rat on a heating pad until waking up from the anesthesia.

n. Observe the rat's health after the awakening.

60. Allow at least 2 weeks for transgene expression.

Detection of transgene expression

Timing: 7 days

In this section, we describe the methods for perfusion, brain sectioning, and double immunofluorescence histochemistry.

61. Perfuse the rat ~2 weeks after vector injection for fixation.
    a. Anesthetize the rat i.p. with the mixed anesthetic solution including medetomidine (0.04 mg/kg), midazolam (0.4 mg/kg) and butorphanol (0.4 mg/kg).
    b. Place the rat in the sealed anesthesia induction chamber and supply 4% isoflurane for 3–5 min.
    c. Move the rat on a perfusion plate and open the thoracic cavity with thin scissors to expose the heart.
    d. Insert a 25-gauge needle into the left ventricle and make a small incision in the right atrium with the scissors.
    e. Perfuse 200 mL of ice-cold 0.1 M PB with a peristaltic pump.

    Note: 0.1 M PB should be stored at 4°C within 1 year.

    f. Perfuse 200 mL of ice-cold 10% formalin neutral buffer solution at a flow rate of 10 mL/min.
    g. Remove the brain and immerse it in 10% formalin solution at 4°C for 16–24 h.
    h. Replace the formalin solution by 10% sucrose in PBS and allow the brain to equilibrate completely at 4°C for 1 day.
    i. Replace with 20% sucrose in PBS and shake gently at 4°C for 1–2 days until the brain is completely submerged.
    j. Repeat similarly with 30% sucrose in PBS.
62. Make sections of the brain tissue.
    a. Cut off the brainstem so that the hemispheres are flattened in the case of coronal sectioning.

b. Place a drop of OCT compound on the fixation plate in a chilled cryostat and attach the brain to the plate, directing the olfactory bulb upwards.
c. Pour carefully the OCT compound over the brain.
d. Leave the embedded brain in the cryostat for 20–30 min.
e. Attach the plate with the brain to the holder of the cryostat.
f. Section the brain into 30-μm thickness (through the striatum) and transfer the sections into PBS with a paintbrush.

63. Incubate the sections with the primary antibodies and species-specific secondary antibodies conjugated to Alexa 488 or Cy3.
    a. Transfer the sections into 5% normal swine serum in PBS and block nonspecific binding for 30–60 min at 24°C–26°C.
    b. Incubate the sections for 12–24 h at 4°C with goat polyclonal anti-GFP antibody (1:4000 dilution) and rabbit polyclonal anti-PV antibody (1:200 dilution) in 500 μL of PBS containing 0.3% Triton-X.
    c. Wash the sections in PBS for 5 min 3 times
    d. Incubate the sections for 2 h at 24°C–26°C with Alexa 488-conjugated goat-IgG antibody (1:1000 dilution) and Cy3-conjugated rabbit IgG antibody (1:1000 dilution) in 500 μL PBS containing 0.3% Triton-X.
    e. Wash the sections in PBS for 5 min twice.
    f. Stain the nuclei with 2 mL of 1 μg/mL DAPI in PBS for 5 min at 24°C–26°C.
    g. Wash the sections in PBS for 5 min twice.
    h. Mount the sections on gelatin-coated glass slides.
    i. Apply a coverslip with a mounting medium for fluorescence microscopy.

    Note: The sections can be stored at −20°C for approximately 2 years.

    j. Visualize the signals with a confocal laser-scanning microscope (Nikon A1) equipped with proper filter cube specifications. See troubleshooting 3 and 4.

64. Count the number of immunostained cells in the regions of interest.
    a. The efficiency of transgene expression in target cell types is evaluated by the percentage of the number of PV^+ + GFP^+ cells divided by the total number of PV^+ cells.
    b. The selectivity of transgene expression in target cell types is validated by the percentage of the number of PV^- + GFP^+ cells divided by the total number of GFP^+ cells.

EXPECTED OUTCOMES

Although there are several evidences for non-specific expression of transgenes in off-target cell populations after injection of AAV vectors carrying the FLEX system into the tissues, studies of the molecular mechanism that produces the non-specific transgene expression were limited.[9] Our recent study has shown that the unexpected Cre-independent recombination in the conventional FLEX structure occurred in AAV viral genome mainly during the production of the vector in packaging cells.[1] To resolve this issue, the study has shown that introducing the USS between the *lox*P and *lox*2272 sites at either the 5′- or 3′-end of the inverted transgene, in particular at the 3′-end, protected Cre-independent recombination in AAV genome during the vector production and non-specific transgene expression after the vector injection, resulting in enhanced selectivity of transgene expression in knockin rats, in which Cre recombinase is expressed in target cell types.[1] The FLEX/USS system provides a powerful tool for highly specific Cre-dependent transgene expression for the labeling of target cell populations and functional imaging, as well as the manipulation of the activity of these populations in the future.

LIMITATIONS

AAV vectors have a size capacity of transgene constructs suitable for the packaging of viral genome. The size of the constructs including the promoter, transgene, WPRE (~0.6 kb), and poly A signal

sequence (~0.5 kb) should be less than 5 kb. If we use the CAG promoter (~1.7 kb), the upper limit for the length of transgene is ~2.2 kb. Replacing the CAG promoter with EF1α promoter (~1.1 kb) can increase the transgene size. However, our system requires the insertion of the USS of 750 bp between two double recognition sites, which thereby may restrict the transgene size.

The selective transgene expression mediated by our FLEX/USS system is dependent on cell type-specific Cre expression in the tissues. In the Cre driver transgenic animals, we occasionally observe a low level of Cre expression in off-target cells, which cannot be detected by immunohistochemistry with anti-Cre antibody, but this expression results in the transgene expression in off-targets, decreasing the selectivity of transgene expression in the cell types of interests. We need the detailed analysis of the patterns of Cre expression in the driver animals, which are used for vector injection with the FLEX/USS system.

Our FLEX/USS strategy was applied for the protection of Cre-independent transgene expression in off-target cells after injection with AAV serotype 2 vector. It is unknown whether our system is effective in other serotypes of AAV vector. In addition, another site-specific recombination system is mediated by the yeast Flp recombinase. The application of our USS for the improvement of selectivity of transgene expression through the FLEX system with other recombinase should be investigated in the future.

TROUBLESHOOTING

Problem 1

Low titer of AAV vectors (step 53).

Potential solution

Production of AAV vectors depends on the efficiency of transfection into HEK293T cells and culture condition of the cells. The transfection efficiency by the calcium phosphate co-precipitation method is affected by the pH of the HBSP buffer solution. Adjust the pH of the solution between 7.05 and 7.20. Some lipofection reagents can be used to increase the transfection efficiency. HEK293T cells should be passaged under the condition of approximately 80% confluent. The excessive number of the passage may decrease the efficiency of vector production. If the vector titer is low, replacing with fresh HEK293T cells may improve the efficiency of vector production.

Problem 2

Lack of the accuracy of vector titer obtained from the real-time quantitative PCR, due to exceeded upper limit of the standard amplification curves (step 53).

Potential solution

The final product of AAV vectors usually has the titers ranging from 10^{12} to 10^{13} genome copies/mL The vector titers vary occasionally dependent on the transgene constructs or serotypes. Dilutions of the vector solution should be checked to obtain the accurate values by the quantitative PCR.

Problem 3

Cellular damage around the injection sites of AAV vectors (step 63j).

Potential solution

Contamination of cell debris or proteins in the vector solution may damage the tissues around the injection sites. To remove these contaminants, the purification processes of the vectors should be done as described. In addition, in our experience the use of the titer higher than 1×10^{13} copies/mL cause the cytotoxicity around the injection sites. Dilute the solution to less than 5×10^{12} copies/mL in PBS before intracranial injection to avoid the cytotoxic effects.

Problem 4

High background or low level of positive signals in brain sections (step 63j).

Potential solution

The specificity and cross-reactivity of the primary and secondary antibodies used for immunostaining should be confirmed. A series of diluted antibody solutions should be checked to reduce the background level and increase the signals specific for the antibodies.

Problem 5

Ectopic transgene expression in non-target cells (step 64b).

Potential solution

Unexpected ectopic transgene expression may be observed in off-target cells, even though leakage was suppressed by using the FLEX/USS system. This is due to the expression of Cre recombinase in the off-target cells in the transgenic animals used in the experiments. The expression patterns of Cre recombinase should be carefully studied and clarified in advance. It is important to ensure that the transgenic animals are appropriate for the purpose of the experiments.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to the lead contact, Kazuto Kobayashi (kazuto@fmu.ac.jp).

Materials availability

This study did not generate new unique reagents, cells, AAV vectors, or transgenic rat strains.

Data and code availability

This study did not generate a new dataset.

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AUTHOR CONTRIBUTIONS

N.M., S.K., and K.K. conceived the study, designed the experiments, and directed the project. N.M., S.K., K.T., and K.K. designed the vector expression strategy and generated the vector. N.M., Y.M., and T.M. produced and analyzed knockin transgenic rats. N.M., S.K., and K.T. analyzed vector structure and K.N. and M.S. performed intracranial injections and histological examinations. N.M., S.K., and K.K. wrote the paper. All authors discussed the protocols and implications and commented on the manuscript at all stages.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Matsushita, N., Kato, S., Nishizawa, K., Sugawara, M., Takeuchi, K., Miyasaka, Y., Mashimo, T., and Kobayashi, K. (2023). Highly selective transgene expression through the flip-excision switch system by using a unilateral spacer sequence. Cell Rep. Methods *3*, 100393. https://doi.org/10.1016/j.crmeth.2022.100393.

2. Schnütgen, F., Doerflinger, N., Calléja, C., Wendling, O., Chambon, P., and Ghyselinck, N.B. (2003). A directional strategy for monitoring Cre-mediated recombination at the cellular level in the mouse. Nat. Biotechnol. *21*, 562–565. https://doi.org/10.1038/nbt811.

3. Sohal, V.S., Zhang, F., Yizhar, O., and Deisseroth, K. (2009). Parvalbumin neurons and gamma rhythms enhance cortical circuit performance. Nature *459*, 698–702. https://doi.org/10.1038/nature07991.

4. Saunders, A., Johnson, C.A., and Sabatini, B.L. (2012). Novel recombinant adeno-associated viruses for Cre activated and inactivated transgene expression in neurons. Front. Neural Circuits *6*, 47. https://doi.org/10.3389/fncir.2012.00047.

5. Kato, S., Fukabori, R., Nishizawa, K., Okada, K., Yoshioka, N., Sugawara, M., Maejima, Y., Shimomura, K., Okamoto, M., Eifuku, S., and

Kobayashi, K. (2018). Action selection and flexible switching controlled by the intralaminar thalamic neurons. Cell Rep. *22*, 2370–2382. https://doi.org/10.1016/j.cellrep.2018.02.016.

6. Tschida, K., Michael, V., Takatoh, J., Han, B.X., Zhao, S., Sakurai, K., Mooney, R., and Wang, F. (2019). A specialized neural circuit gates social vocalizations in the mouse. Neuron *103*, 459–472.e4. https://doi.org/10.1016/j.neuron.2019.05.025.

7. Woods, N.I., Stefanini, F., Apodaca-Montano, D.L., Tan, I.M.C., Biane, J.S., and Kheirbek, M.A. (2020). The dentate gyrus classifies cortical representations of learned stimuli. Neuron *107*, 173–184.e6. https://doi.org/10.1016/j.neuron.2020.04.002.

8. Matsushita, N., Nishizawa, K., Kato, S., Iguchi, Y., Fukabori, R., Takeuchi, K., Miyasaka, Y., Mashimo, T., and Kobayashi, K. (2022). Catecholaminergic cell type-specific expression of Cre recombinase in knock-in transgenic rats generated by the Combi-CRISPR technology. J. Neurosci. Methods *381*, 109707. https://doi.org/10.1016/j.jneumeth.2022.109707.

9. Fischer, K.B., Collins, H.K., and Callaway, E.M. (2019). Sources of off-target expression from recombinase-dependent AAV vectors and mitigation with cross-over insensitive ATG-out vectors. Proc. Natl. Acad. Sci. USA *116*, 27001–27010. https://doi.org/10.1073/pnas.1915974116.

10. Grieger, J.C., Choi, V.W., and Samulski, R.J. (2006). Production and characterization of adeno-associated viral vectors. Nat. Protoc. *1*, 1412–1428. https://doi.org/10.1038/nprot.2006.207.

11. Courtland, J.L., Bradshaw, T.W., Waitt, G., Soderblom, E.J., Ho, T., Rajab, A., Vancini, R., Kim, I.H., and Soderling, S.H. (2021). Genetic disruption of WASHC4 drives endo-lysosomal dysfunction and cognitive-movement impairments in mice and humans. Elife *10*, e61590. https://doi.org/10.7554/eLife.61590.

12. Paxinos, G., and Watson, C. (2007). The Rat Brain in Stereotaxic Coordinates, Ed 6 (Academic Press).