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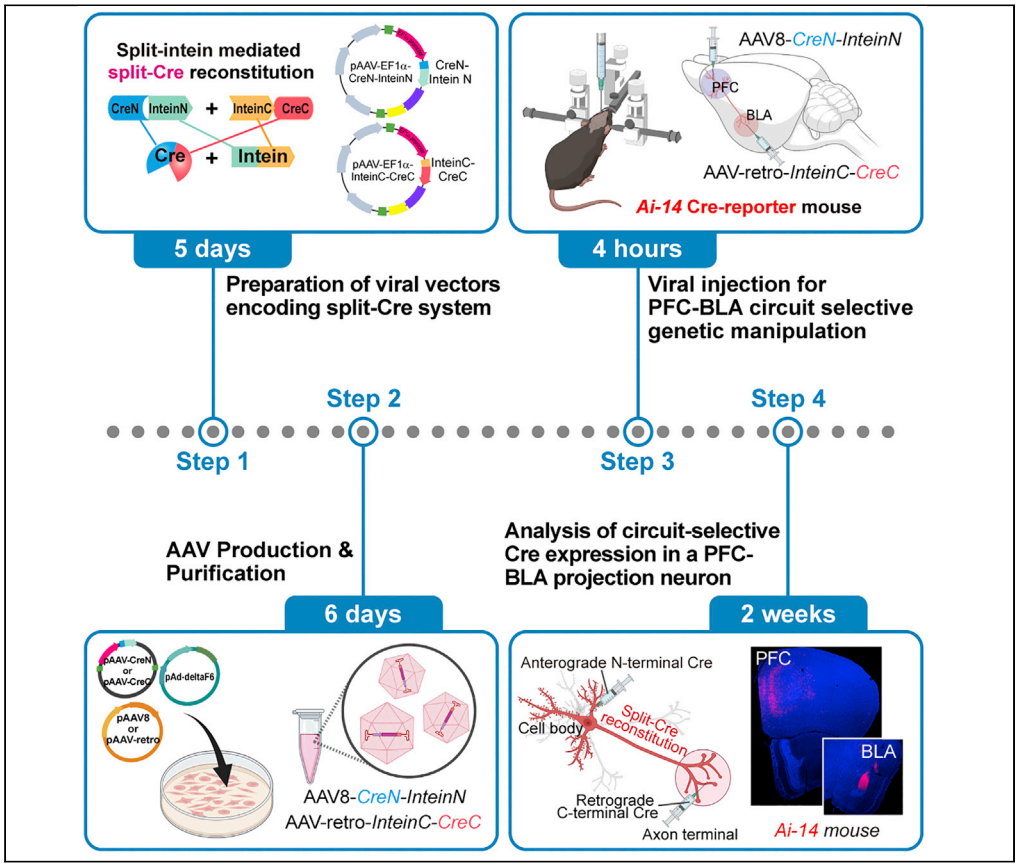
Neural circuit-specific gene manipulation in mouse brain *in vivo* using split-intein-mediated split-Cre system



Neural network studies require efficient genetic tools to analyze individual neural circuit functions *in vivo*. Thus, we developed an advanced circuit-selective gene manipulating tool utilizing anterograde and retrograde adeno-associated viruses (AAVs) encoding split-intein-mediated split-Cre. This strategy can be applied to visualize a specific neural circuit as well as manipulate multiple genes in the circuit neurons. Here, we describe the production and purification of the AAVs, viral injection to the mouse brain, and imaging analysis for a specific neural circuit.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

In vivo application of the split-intein/split-Cre system using mouse models

Split-Cre reconstitution system for neural circuit-specific gene manipulation

Robust and highly specific unidirectional neural circuit-selective Cre expression

Simultaneous manipulation of multiple genes in a specific unidirectional neural circuit

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Neural circuit-specific gene manipulation in mouse brain *in vivo* using split-intein-mediated split-Cre system

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SUMMARY

Neural network studies require efficient genetic tools to analyze individual neural circuit functions *in vivo*. Thus, we developed an advanced circuit-selective gene manipulating tool utilizing anterograde and retrograde adeno-associated viruses (AAVs) encoding split-intein-mediated split-Cre. This strategy can be applied to visualize a specific neural circuit as well as manipulate multiple genes in the circuit neurons. Here, we describe the production and purification of the AAVs, viral injection to the mouse brain, and imaging analysis for a specific neural circuit.

For complete details on the use and execution of this protocol, please refer to Kim et al. (2022).

BEFORE YOU BEGIN

The brain functions through a complex neural network interconnection between various brain regions. To study the neural network, a precise genetic manipulation tool with high selectivity for a specific neural circuit is mandated. The Cre-LoxP system has been the most widely utilized tool for genetic manipulation that deletes or expresses target genes. Multiple strategies have been developed to express Cre in specific brain regions or cell types (Feil et al., 1996). Recently, we developed a novel method expressing Cre recombinase exclusively within a unidirectional projection circuit (Kim et al., 2022). This strategy combines a split-Intein mediated split-Cre reconstitution system (Evans et al., 2000; Wang et al., 2012; Wu et al., 1998) (Figure 1A) with a serotype-dependent bidirectional gene delivery mechanism (Haery et al., 2019; Tervo et al., 2016). In brief, we anterogradely express a fusion protein that encodes N-terminal Cre (CreN; amino acids 19–59) and N-terminal Intein (InteinN) in the cell body region using an AAV8 serotype (AAV8-*CreN-InteinN*). Meanwhile, we retrogradely express a fusion protein encoding C-terminal Cre (CreC; amino acids 60–343) and C-terminal Intein (InteinC) in the axonal terminal region using AAV-retro (AAV-retro-*InteinC-CreC*). Using this strategy, we can express full-length Cre exclusively in a unidirectional neural circuit. This simple but powerful strategy can be utilized for circuit-selective gene deletion or expression (Figure 1B).

Here, we provide a detailed protocol for preparation/purification of the AAVs, injection of the AAVs into the brain, and analysis of the histological data.

> ***Note:*** We have already completed the production of the pAAV-*Ef1α-CreN-InteinN* and pAAV-*Ef1α-InteinC-CreC* plasmids (Figure 2). The plasmids and their sequence information have been deposited and are now available in the Addgene (Addgene ID: 187614, 187615). The reconstitution capability of these plasmids can be validated in HEK293T cells as below.

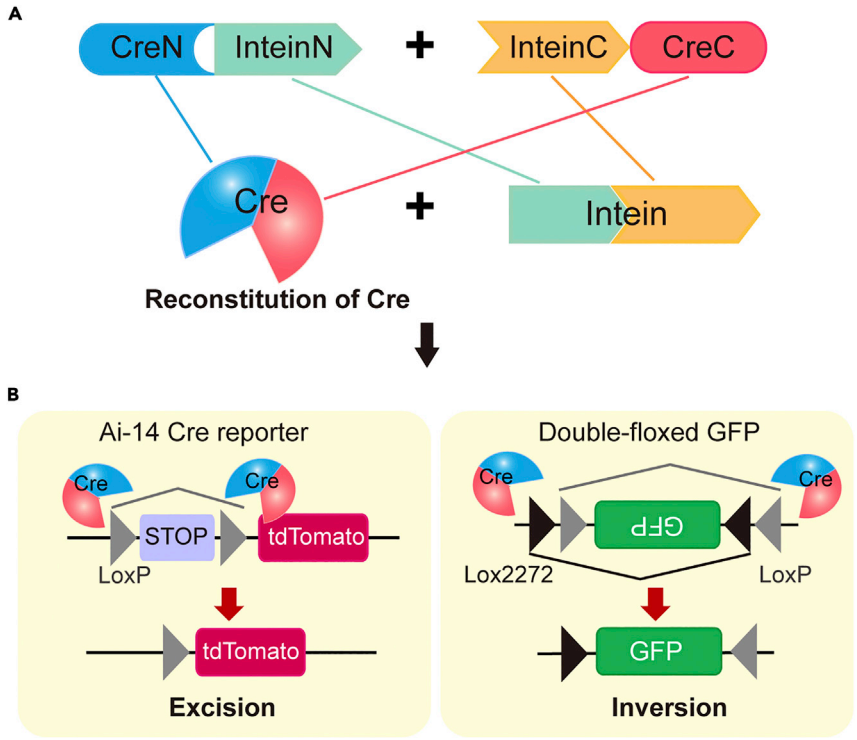

Figure 1. Schematic diagram of split-Intein-mediated split-Cre reconstitution

(A) The N-terminal Cre (CreN) and C-terminal Cre (CreC) expressed by each promoter are reconstituted to form a full-length Cre recombinase by Intein-mediated protein splicing.

(B) In *Ai-14* Cre reporter mice, the STOP cassette is excised by reconstituted Cre, leading to the expression of tdTomato protein. The double-floxed GFP reporter gene with inverted orientation is corrected by the reconstituted Cre to express GFP protein.

Validating Cre reconstitution capability of the pAAV-*Ef1α-CreN-InteinN* and pAAV-*Ef1α-InteinC-CreC*

Timing: 3 days

1. Seed HEK293T cells in a 4-well Chamber Slide at a density of 5 × 10^5 cells/well that contains 500 μL of DMEM/F-12 supplemented with 10% FBS and 1× penicillin-streptomycin, and then incubate at 37°C overnight (16–24 h) in a 5% CO_2 humidified incubator.
2. Prepare the combinational mixtures of plasmids [pAAV-*Ef1α-CreN-InteinN*, pAAV-*Ef1α-InteinC-CreC*, and AAV-*Ef1α-flex-GFP* (Cre reporter)] as follows.
   a. As shown in the table below, dilute plasmids in Opti-MEM to a total volume of 50 μL in 1.5 mL microtube A (tube A). Vortex gently and spin down briefly.

Plasmid dilution in tube A, related to Figure 3.

DNA	Control	CreN-InteinN	InteinC-CreC	CreN-InteinN & InteinC-CreC
pAAV-*Ef1α-CreN-inteinN*	–	0.5 μg	–	0.5 μg
pAAV-*Ef1α-inteinC-CreC*	–	–	0.5 μg	0.5 μg
AAV-*Ef1α-flex-GFP*	0.5 μg	0.5 μg	0.5 μg	0.5 μg

   b. Add 8.25 μL of PEI into 1.5 mL microtube B (tube B) with 41.75 μL of Opti-MEM solution. Vortex and spin down briefly.
   c. Incubate the diluted plasmids (tube A) and PEI solution (tube B) in RT for 10 min.

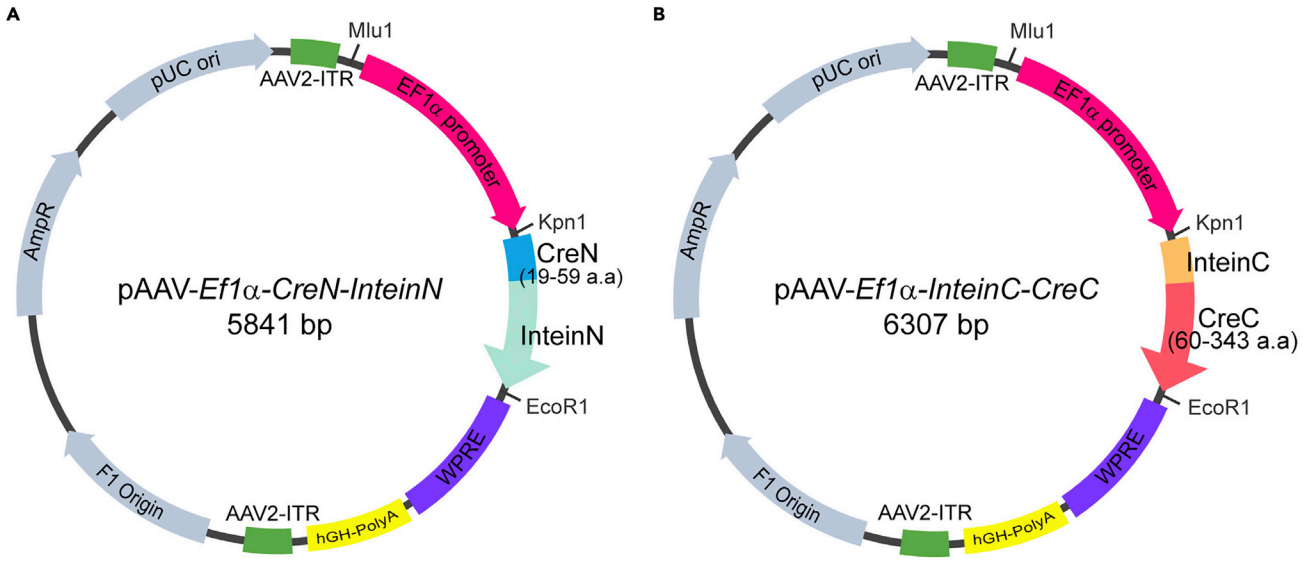

Figure 2. Maps of the pAAV plasmids carrying split-Intein-mediated split-Cre
(A and B) Maps of the pAAV-*Ef1α-CreN-InteinN* (A) and pAAV-*Ef1α-InteinC-CreC* (B) plasmid.

d. Mix the diluted plasmids (tube A) and PEI solution (tube B), and then vortex thoroughly and spin down briefly.
e. Incubate the plasmids/PEI mixture for 20 min at RT.

3. Add 100 μL of the mixture (plasmids and PEI) dropwise to each well of the Chamber Slide plate, and shake gently.
4. After 6–12 h of transfection, aspirate the media in the Chamber Slide plate, and add the fresh 500 μL of HEK293T cell culture media (DMEM/F-12 supplemented with 10% FBS and 1× penicillin-streptomycin) to each chamber.
5. Incubate the transfected HEK293T cells at 37°C in a 5% CO_2 humidified incubator for two days.
6. After aspirating the HEK293T cell culture media, fix the HEK293T cells by adding 300 μL of 4% paraformaldehyde (PFA) in PBS at RT for 10 min.
7. After aspirating the 4% PFA solution, add 300 μL of 0.2% TritonX-100 in PBS into the HEK293T cells and incubate at RT for 15 min.
8. After aspirating the 0.2% TritonX-100 solution, stain the cell nuclei by adding 300 μL of 1 μg/mL 4′,6′-diamidino-2-phenylindole (DAPI) solution in PBS for 10 min.
9. Wash the HEK293T cells thrice with 300 μL of 0.2% TritonX-100 solution in PBS.
10. Remove the medium chamber from the slide plate.
11. Apply a small drop of Prolong™ glass antifade mountant to the surface of the slide and place a coverslip over mountant.
12. Take images of the samples using a confocal or fluorescent microscope (here we used CELENA-S Digital Imaging System from Logos Biosystem), and check GFP and DAPI signals.

As a result, the GFP protein is expressed only when the pAAV-*Ef1α-CreN-InteinN* and *pAAV-Ef1α-InteinC-CreC* plasmids are co-expressed with AAV-*Ef1α-Flex-GFP* (Figure 3), indicating that CreN-InteinN and InteinC-CreC are successfully reconstituted to express full-length Cre in the HEK293T cells. These data also confirm that a partial Cre (CreN or CreC) has no Cre function.

Institutional permissions

All procedures for animal experiments were performed with a protocol approved by the University of Tennessee Institutional Animal Care and Use Committee in accordance with US National Institutes of Health guidelines.

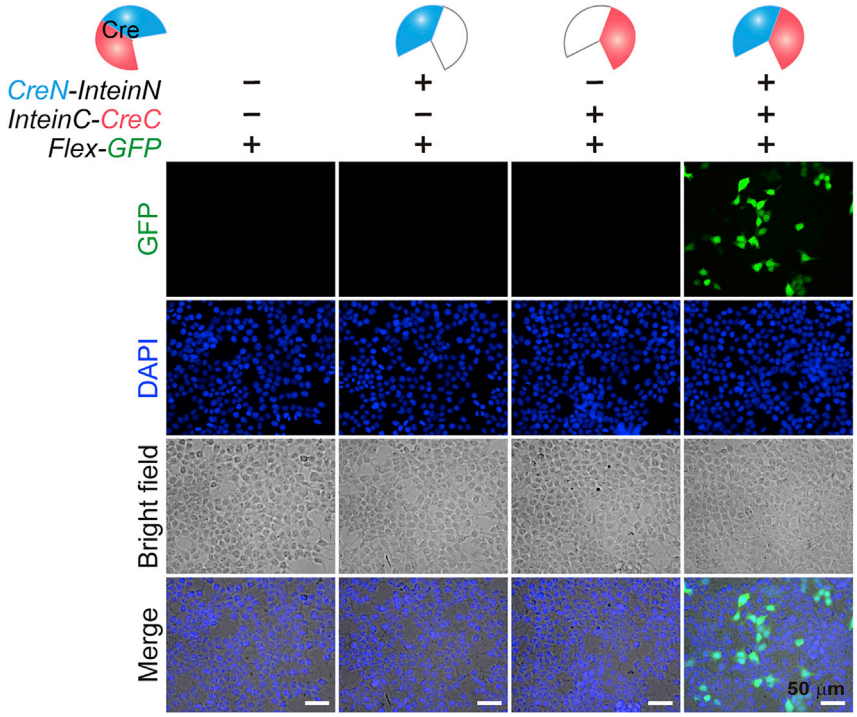

Figure 3. Validation of split-Cre reconstitution using HEK293T cells

Combinational transfections of pAAV-*Ef1α-CreN-inteinN*, pAAV-*Ef1α-inteinC-CreC*, and AAV-*Ef1α-flex-GFP* plasmids into HEK293T cells reveal that GFP is expressed only in the cells transfected with all the three plasmids. Scale bar, 50 μm.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
AAV8-*CreN-InteinN*	(Kim et al., 2022) https://doi.org/10.1016/j.celrep.2022.110906	N/A
AAV-retro-*InteinC-CreC*	(Kim et al., 2022) https://doi.org/10.1016/j.celrep.2022.110906	N/A
Escherichia coli; Stbl3	Thermo Fisher Scientific	C737303
Chemicals, peptides, and recombinant proteins		
MluI-HF	New England Biolabs	R3198S
Kpn1-HF	New England Biolabs	R3142S
T4 DNA ligase	Thermo Scientific	EL0014
Carbenicillin solution (100 mg/mL)	Teknova	C2130
LB Lennox broth	IBI Scientific	IB49112
Bacteriological agar	IBI Scientific	IB49171
DMEM Nutrient mix F12	Gibco	11320082
DPBS, no calcium, no magnesium	Thermo Scientific	14190250
Heat Inactivated Fetal Bovine Serum	Gibco	10438026
Penicillin Streptomycin Solution	Gibco	15140122
Trypsin 0.05% EDTA	Corning	25300054
Opti-MEM	Gibco	31985070
PEI	Polysciences	24765
OptiPrep™ Density Gradient Medium (Iodixanol)	Sigma	D1556
Benzonase	Novagen	70664

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Phenol red solution	Sigma	P0290
4,6-diamidino-2-phenylindole (DAPI) solution (1 mg/mL)	Thermo Scientific	62248
SYBR™ Select Master Mix for CFX	Thermo Scientific	4472942
Isoflurane	Henry Schein	1182097
Levafen (Carprofen injection, solution)	Pivetal	NDC46066-936-01
0.5% Bupivacaine Hydrochloride injection (250 mg/50 mL)	Pfizer Inc	NDC0409-1163-18
LubriFresh P.M (Eye lubricant)	Major Pharmaceuticals	NDC 0904-6488-38
10% Povidone-iodine	Betadine	995292
Mineral oil	Sigma-Aldrich	M5904
SporGon Sporicidal Disinfectant	Decon	4301
Tris	IBI	IB70145
Sodium chloride (NaCl)	Sigma-Aldrich	S9888
Potassium chloride (KCl)	Sigma-Aldrich	P3911
Sodium phosphate dibasic (Na_2HPO_4)	Sigma-Aldrich	S9763
Potassium phosphate monobasic (KH_2PO_4)	Sigma-Aldrich	P0662
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich	M8266
Sodium hydroxide (NaOH), Pellets	Fisher Chemical	S318-500
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	E9884
Paraformaldehyde	Electron Microscopy Sciences	19202
TritonX-100	Sigma-Aldrich	X100
Sucrose	IBI	IB37165
Heparin, Sodium salt	Millipore	375095
Tissue-Tek® O.C.T. Compound	Sakura Finetek USA INC	4583
ProLong™ Glass Antifade Mountant	Invitrogen	P36984
UltraPure™ DNase/RNase-Free Distilled Water	Invitrogen	10977015
Critical commercial assays		
PureLink™ HiPure Plasmid Filter Maxiprep Kit	Invitrogen	K210017
PureLink™ Quick Plasmid Miniprep Kit	Invitrogen	K210011
Gel Extraction Kit	Thermo Fisher Scientific	K0691
Experimental models: Cell lines		
HEK293T (Use 10–15 passages)	ATCC	CRL-11268
Experimental models: Organisms/strains		
Mouse: *Ai-14* (B6;129S6-Gt(ROSA) 26Sortm14(CAG-tdTomato)Hze/J) (Male mouse aged 8 weeks)	The Jackson Laboratory	#0007908
Oligonucleotides		
WPRE-F	IDT	5′-TGG CGT GGT GTG CAC TGT-3′
WPRE-R	IDT	5′-AGG GAC GTA GCA GAA GGA CG-3′
Recombinant DNA		
pAAV-*Ef1α-CreN-InteinN*	(Kim et al., 2022) https://doi.org/10.1016/j.celrep.2022.110906	Addgene plasmid # 187614
pAAV-*Ef1α-InteinC-CreC*	(Kim et al., 2022) https://doi.org/10.1016/j.celrep.2022.110906	Addgene plasmid # 187615
AAV-*Ef1α-Flex-GFP*	(Kim et al., 2020) https://doi.org/10.1016/j.celrep.2020.107965	N/A
AAV2/8	Addgene, Deposited by James M. Wilson (RRID: Addgene_112864)	Addgene plasmid # 112864
AAV-retro helper	Addgene, (Tervo et al., 2016) https://doi.org/10.1016/j.neuron.2016.09.021	Addgene plasmid # 81070
pAd-deltaF6	Addgene, Deposited by James M. Wilson (RRID: Addgene_112867)	Addgene plasmid # 112867
Software and algorithms		
iQ5 optical system software	Bio-Rad	https://www.bio-rad.com/en-us/sku/1709753-iq5-optical-system-software?ID=1709753

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Zen software	ZEISS	https://www.zeiss.com/microscopy/en/products/software/zeiss-zen.html
Other		
Fisherbrand™ Petri Dishes with Clear Lid	Fisherbrand	FB0875713
Fisherbrand™ Surface Treated Tissue Culture Dishes	Fisherbrand	FB012925
12 well plate	USA Scientific	CC7682-7512
Nunc™ Lab-Tek™ II Chamber Slide™ System (4-well Chamber Slide)	Thermo Scientific	154526
Axygen™ MaxyClear Snaplock Microtubes, 1.5 mL	Axygen	MCT-150-C
Cell Lifter	Corning	3008
Pasteur pipets	Fisherbrand	13-678-6C
Amicon™ Ultra-15 Centrifugal Filter Units	MilliporeSigma	UFC910024
OptiSeal Tube	Beckman Coulter	361625
0.2 mL 8-Tube PCR Strips without Caps low profileclear	Bio-Rad	TLS0801
0.2 mL Flat PCR Tube 8-Cap Strips optical ultraclear	Bio-Rad	TLS0803
Sutter Instrument Borosilicate glass with filament	Sutter Instrument Co.	BF120-94-10
Microscope slides (Superfrost® Plus Micro Slide)	VWR	48311-703
Cover glass	Epredia	102250
Tubing	Tygon	R-3603
10 mL syringe (BD Luer-Lok tip)	BD	309604
PrecisionGlide 19G × 1 1/2″ Needle	BD	305187
PrecisionGlide 30G × 1/2″ Needle	BD	305106
25 Gauge needle (butterfly infusion set)	Med-Vet International	26708
MicroFil 28 Gauge/97 mm long	World Precision Instruments, Inc	MF28G-5
Cotton swabs	McKesson	24-103
Gelfoam®	Pharmacia & Upjohn	NDC 0009-0396-01
Dumont # 5 Forceps	Fine Science Tools	1125130
Fine scissors (Sharp)	Fine Science Tools	1406111
Perma-hand Silk Suture	Med-Vet International	683G
Heating pad	Sunbeam	Size: 12 × 15-Inch
Tabletop Laboratory Animal Anesthesia System (containing VaporGuard filter)	VetEquip, Inc	901806
Dual Ultra Precise Small Animal Stereotaxic Instrument	David Kopf Instruments	Model 962
Ultra Precise Micro Manipulator	David Kopf Instruments	Model 961
Mouse Gas Anesthesia Head Holder	David Kopf Instruments	Model 923-B
Non-Rupture Ear Bars, mouse	David Kopf Instruments	Model 922
Electric hair clipper	Wahl	–
High-Speed Stereotaxic Drill (MH-170 Handpiece) and holder	David Kopf Instruments	Model 1474
Round operative carbide bur (0.5 MM HP)	Meisinger	US#1/4
Nanoject II microinjector	Drummond Scientific	3-000-204
Leica Wild M691 Surgical Microscope	Leica	Wild M691
Masterflex® Peristaltic Tubing Pumps	Cole-Parmer	7553-80, 7519-20, 7519-75, 7553-71
Shaker (Talboys™ Standard Analog 1000 Orbital Shaker)	Talboys	980173
Beckman L8-70M Ultracentrifuge	Beckman Coulter	L8-70M
Type 70 Ti Fixed-Angle Titanium Rotor	Beckman Coulter	337922
Sorvall LYNX 6000 Superspeed Centrifuge	Thermo Scientific	75006590
Sorvall Legend Micro 21R	Thermo Scientific	75002446
IQ5 Real-Time PCR Complete system	Bio-Rad	IQ5

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
P-97 Flaming/Brown Micropipette Puller	Sutter Instrument Co.	P-97
Biosafety cabinet (1300 Series Class II, Type A2)	Thermo Scientific	1385
Forma™ Steri-Cycle™ CO_2 incubator	Thermo Scientific	370
Shaking incubators	Eppendorf	New Brunswick™ Excella® E25
Isotemp™ microbiological Incubator	Fisherbrand	151030513
Cryostat	Leica Biosystems	Leica CM1950
CELENA® S Digital Imaging System	Logos Biosystems	CS20001
Confocal Microscope	ZEISS	LSM 710

MATERIALS AND EQUIPMENT

LB Broth

Reagent	Amount
LB Lennox Broth	20 g
double-distilled H_2O (ddH_2O)	~ 950 mL
Total	**1 L**

Adjust the volume to 1 L with additional ddH_2O. Autoclave at 121°C for 15 min and store at room temperature (20°C–22°C) for up to 6 months.

LB agar plate containing 100 μg/mL carbenicillin

Reagent	Amount
LB Lennox Broth	10 g
Bacteriological agar	7.5 g
double-distilled H_2O (ddH_2O)	~ 450 mL
Total	**500 mL**

Adjust the volume to 500 mL with additional ddH_2O. After autoclaving at 121°C for 15 min, leave the LB agar solution to cool to approximately 55°C. Add the 500 μL of carbenicillin solution (100 mg/ mL) into the LB agar solution and swirl to mix. Pour ~ 15 mL of LB agar solution into 10 cm Petri dish and cover the plates with a lid. Cool until solidified. Invert the LB agar plates and store them in plastic bags at 4°C for up to 2 months.

HEK293T cell culture media

Reagent	Final concentration	Volume
DMEM Nutrient mix F12	N/A	445 mL
Heat Inactivated Fetal Bovine Serum (HI-FBS)	10%	50 mL
Penicillin Streptomycin Solution (100×)	1×	5 mL
Total		**500 mL**

Store at 4°C for up to 6 months.

1× PBS buffer

Reagent	Final concentration	Amount
NaCl	137 mM	8 g
KCl	2.7 mM	0.2 g
Na_2HPO_4	10 mM	1.44 g
KH_2PO_4	1.8 mM	0.24 g
double-distilled H_2O (ddH_2O)	N/A	~ 900 mL
Total		**1 L**

Adjust pH to 7.4 and adjust the volume to 1 L with additional ddH_2O. Sterilize by passing through a 0.22 μm filter and store at room temperature (20°C–22°C) for up to 1 year.

1 M NaCl/PBS-MK buffer		
Reagent	Final concentration	Amount
NaCl	1 M	58.4 g
$MgCl_2$	2.76 mM	263 mg
KCl	2 mM	149.1 mg
1× PBS	1×	~ 900 mL
Total		**1 L**

Adjust the volume to 1 L with 1× PBS. Sterilize by passing through a 0.22 μm filter and store at 4°C for up to 1 year.

PBS-MK buffer		
Reagent	Final concentration	Amount
$MgCl_2$	2.76 mM	263 mg
KCl	2 mM	149.1 mg
1× PBS	1×	~ 950 mL
Total		**1 L**

Adjust the volume to 1 L with 1× PBS. Sterilize by passing through a 0.22 μm filter and store at 4°C for up to 1 year.

1 M Tris-HCl (pH 8.5) / 1 M Tris-HCl (pH 5.0)	
Reagent	Amount
Tris	121.14 g
ddH_2O	~ 900 mL
Total	**1 L**

Adjust pH to 8.5 or pH to 5.0 by adding HCl solution and then adjust the volume to 1 L with ddH_2O. Sterilize by passing through a 0.22 μm filter and store at room temperature (20°C–22°C) for up to 1 year.

Cell lysis buffer		
Reagent	Final concentration	Volume
1 M Tris-HCl (pH 8.5),	50 mM	50 mL
5 M NaCl	150 mM	30 mL
ddH_2O	N/A	~ 910 mL
Total		**1 L**

Adjust pH to 8.5 and adjust the volume to 1 L with ddH_2O. Sterilize by passing through a 0.22 μm filter and store at 4°C for up to 1 year.

PEI solution (0.6 mg/mL, transfection reagent)	
Reagent	Amount
PEI (Molecular weight 40 kDa)	0.15 g
ddH_2O (cell culture grade distilled water)	~ 240 mL
Total	**250 mL**

Adjust pH to 7.4 by adding small amounts of a 5 N NaOH solution and adjust the volume to 250 mL with ddH_2O. Sterilize by passing through a 0.22 μm filter. Aliquot 1 mL of PEI solution into 1.5 mL microtube. Store PEI solution at - 20°C for up to 1 year.

Note: Transfection efficiency should be tested for every batch of PEI solutions.

15% Iodixanol solution		
Reagent	Final concentration	Volume
60% Iodixanol	15%	4.5 mL
1 M NaCl/PBS-MK buffer	N/A	13.5 mL
Total		**18 mL**

25% Iodixanol solution		
Reagent	Final concentration	Volume
60% Iodixanol	25%	5 mL
PBS-MK buffer	N/A	7 mL
Phenol red	0.25%	30 μL
Total		**12 mL**

40% Iodixanol solution		
Reagent	Final concentration	Volume
60% Iodixanol	40%	6.7 mL
PBS-MK buffer	N/A	3.3 mL
Total		**10 mL**

60% Iodixanol solution		
Reagent	Final concentration	Volume
60% Iodixanol	60%	10 mL
Phenol red	0.45%	45 μL
Total		**10 mL**
Prepare the fresh solution before use. The volume of iodixanol solution is for two OptiSeal tubes.		

PCR Alkaline digestion buffer		
Reagent	Final concentration	Volume
5 M NaOH	25 mM	0.5 mL
0.5 M EDTA	0.2 mM	0.04 mL
ddH_2O	N/A	99.46 mL
Total		**100 mL**
Store at room temperature (20°C–22°C) for up to 1 year.		

PCR Neutralization buffer		
Reagent	Final concentration	Volume
1 M Tris-HCl (pH 5.0)	40 mM	4 mL
ddH_2O	N/A	96 mL
Total		**100 mL**
Store at room temperature (20°C–22°C) for up to 1 year.		

4% Paraformaldehyde (PFA)	
Reagent	Amount
Paraformaldehyde	40 g
1× PBS	~ 900 mL
Total	**1 L**
Add 1× PBS to a beaker and heat to approximately 60°C. Add approximately 11 NaOH pellets and dissolve with constant stirring. Add the paraformaldehyde and dissolve with constant stirring and heating until the solution is clear. Adjust pH to 7.4 by adding HCl and adjust the volume to 1 L with 1× PBS. Sterilize by passing through a 0.22 μm filter and store at 4°C for at least a month and at - 20°C for up to 1 year.	

25 U/mL heparin in PBS	
Reagent	Amount
Heparin (197.0 U/mg)	126.9 mg
1× PBS	~ 990 mL
Total	**1 L**
Adjust the volume to 1 L with 1× PBS and store at 4°C for up to 1 month.	

30% Sucrose in PBS	
Reagent	Amount
Sucrose	150 g
1× PBS	~ 300 mL
Total	500 mL
Adjust the volume to 500 mL with 1× PBS and store at 4°C for up to 1 month.	

0.2% TritonX-100/PBS	
Reagent	Amount
TritonX-100	1 mL
1× PBS	499 mL
Total	500 mL
Store at 4°C for up to 1 month.	

STEP-BY-STEP METHOD DETAILS

Note: The pAAV-*Ef1α-CreN-InteinN* and pAAV-*Ef1α-InteinC-CreC* plasmids are available in Addgene (Addgene ID: 187614, 187615). If required to replace the Ef1α promoter with other promoters appropriate for their experimental purposes, please refer to Part 1.

If modifications of the pAAV-*Ef1α-CreN-InteinN* and pAAV-*Ef1α-InteinC-CreC* plasmids are not required, skip Part 1 and proceed directly to the production and purification of adeno-associated virus (AAV) (Part 2).

Part 1. Replacing promoters of the plasmids

Timing: 5 days

This section describes a cloning method for replacing the EF1α promoter of pAAV-*Ef1α-CreN-InteinN* and pAAV-*Ef1α-InteinC-CreC* plasmids with other promoters.

Note: The Ef1α promoter can be replaced using the Mlu1 and Kpn1 restriction enzyme sites as illustrated in Figure 2. For example, CMV (cytomegalovirus), CAG (CMV immediate enhancer/chicken β actin), or CBA (modified chicken β actin) promoters can be used as alternatives for ubiquitous expression of transgenes (Gray et al., 2011; McCown et al., 1996; Nathanson et al., 2009). In addition, hSyn1 (human Synapsin 1), CaMKII (Ca^{2+}/Calmodulin-dependent kinase II), or mDLX (mouse DLX5/6 enhancer, minimal promoter and chimeric intron) promoters can be used for specific neuronal expression (Dimidschstein et al., 2016; Hoesche et al., 1993; Nathanson et al., 2009).

1. Cloning of pAAV-*CreN-InteinN* and pAAV-*InteinC-CreC* plasmids containing a specific promoter.
   a. Synthesize the double-stranded specific promoter fragment that is appropriate for researcher's experimental purposes.

Note: To insert a specific promoter into the AAV vectors, the restriction enzyme recognition sequences for Mlu1 (5′-GCGC**ACGCGT**-3′) and Kpn1 (5′-**GGTACC**GCGC-3′) in 5′ and 3′-regions, respectively, must be added to the synthesized promoter DNA fragment. The GCGC sequences are extra base pairs to ensure efficient digestion by restriction enzymes.

b. Incubate 500 ng of the synthesized specific promoter DNA fragment with 1 μL Mlu1 and 1 μL Kpn1 in 1× reaction buffer at 37°C for 1 h.
c. Isolate the specific promoter DNA fragments by agarose gel electrophoresis using a gel extraction kit according to the manufacturer's instructions.
d. Cut each 500 ng of pAAV-*Ef1α-CreN-InteinN* and pAAV-*Ef1α-InteinC-CreC* plasmid with 1 μL Mlu1 and 1 μL Kpn1 in 1× reaction buffer supplied with restriction enzyme at 37°C for 1 h.
e. Isolate and purify the 4,556 bp fragment from pAAV-Ef1α-CreN-InteinN and the 5,022 bp fragment from pAAV-Ef1α-InteinC-CreC plasmids using a gel extraction kit according to the manufacturer's instructions. Discard the 1,275 bp fragment originating from the Ef1α promoter of each plasmid.
f. Insert the synthesized/cut promoter DNA fragment into the cut/linearized pAAV-*CreN-InteinN* or pAAV-*InteinC-CreC* vector using T4 DNA ligase according to the manufacturer's instructions.
g. Transform the ligated plasmids into Stbl3™ *E. coli* strain and spread the transformed Stbl3™ *E. coli* onto an LB agar plate containing 100 μg/mL carbenicillin, and then incubate at 37°C overnight (16–24 h).

Note: We recommend using a competent Stbl3™ *E. coli* strain for the cloning. Stbl3™ *E. coli* carries a recA13 mutation in their genotype, thus reducing the recombination that occurs in cloned DNA containing repeat sequences such as the inverted terminal repeat (ITR) of AAV vector (Al-Allaf et al., 2013).

h. Inoculate a single colony into 5 mL of LB broth containing 100 μg/mL carbenicillin and incubate at 37°C overnight (16–24 h) with shaking.
i. Centrifuge the 5 mL of Stbl3™ *E. coli* at 5,000 × *g* for 5 min at RT (20°C–22°C). Extract and purify the plasmids using PureLink™ Quick Plasmid Miniprep Kit according to the manufacturer's instruction.
j. Identify pAAV-*CreN-InteinN* and pAAV-*InteinC-CreC* plasmids containing a specific promoter with Mlu1 and Kpn1 restriction enzyme and gel electrophoresis.
k. Prepare high-concentrated pAAV-*CreN-InteinN* and pAAV-*InteinC-CreC* plasmids containing a specific promoter for AAV production using Plasmid Filter Maxiprep Kit according to the manufacturer's instructions.

Part 2. Production and purification of the AAVs

Timing: 6 days

Timing for virus production: 5 days for step 2

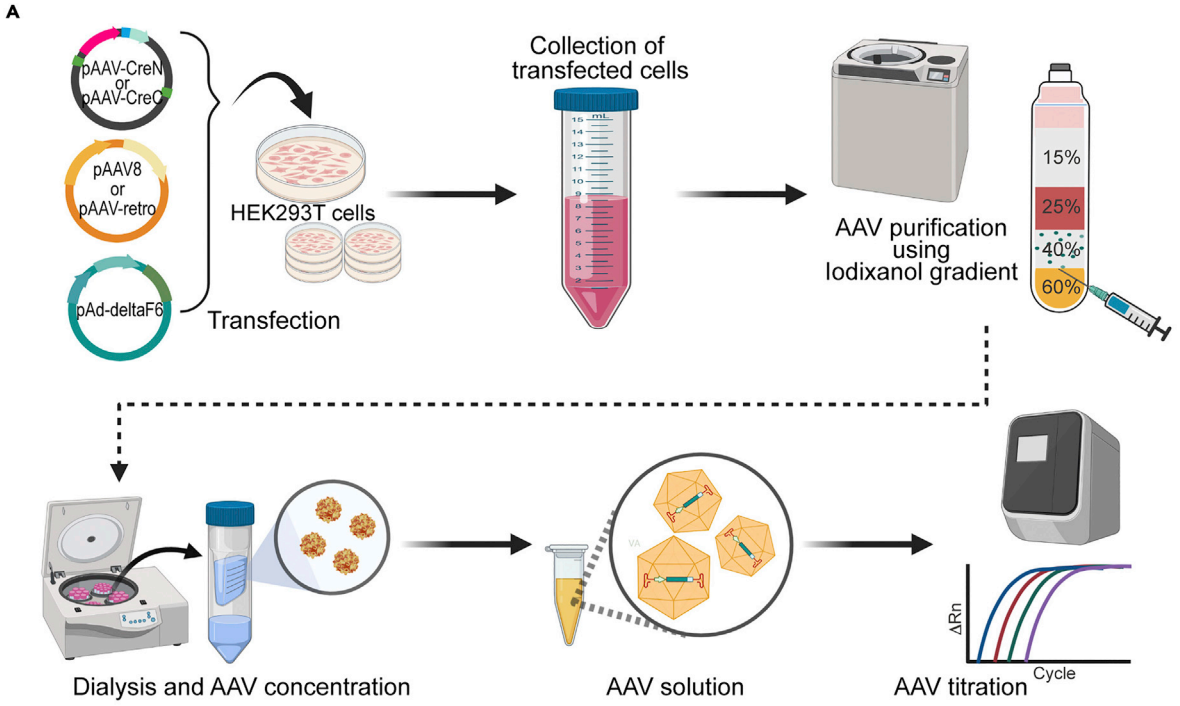
Timing for AAV purification: 9 h for step 5

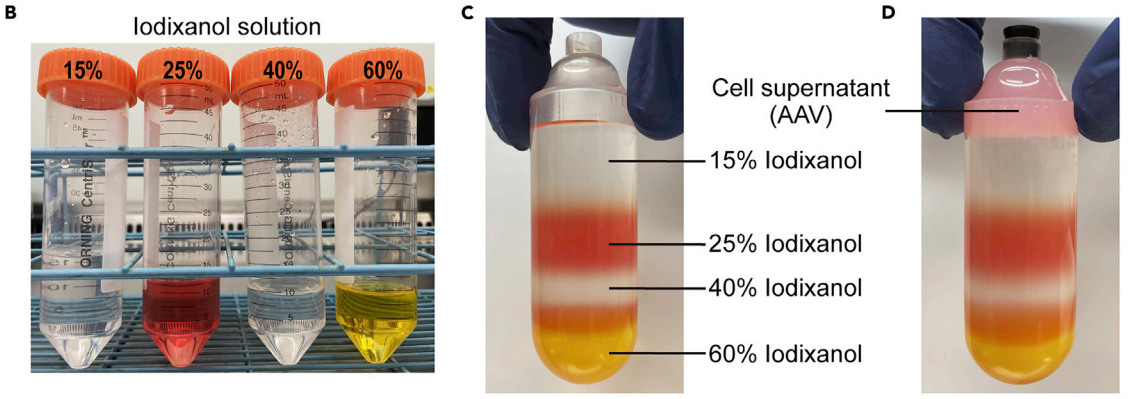
Timing for AAV titration: 5 h for step 7

This section describes how to generate the AAV8-*CreN-InteinN* and AAV-retro-*InteinC-CreC* *via* transfecting plasmids into HEK293T cells, purifying the AAVs by iodixanol gradient ultracentrifugation, and determining the titration of AAVs.

AAV preparation (Figure 4A) follows the previous reports including ours (Courtland et al., 2021; Grieger et al., 2006; Kim et al., 2015, 2020, 2022; Zolotukhin et al., 1999).

2. HEK293T cell culture.


A
pAAV-CreN or pAAV-CreC
pAAV8 or pAAV-retro
pAd-deltaF6
Transfection
HEK293T cells
Collection of transfected cells
AAV purification using Iodixanol gradient
15%
25%
40%
60%
Dialysis and AAV concentration
AAV solution
AAV titration
ΔRn
Cycle


B
Iodixanol solution
15%
25%
40%
60%
C
Cell supernatant (AAV)
15% Iodixanol
25% Iodixanol
40% Iodixanol
60% Iodixanol
D

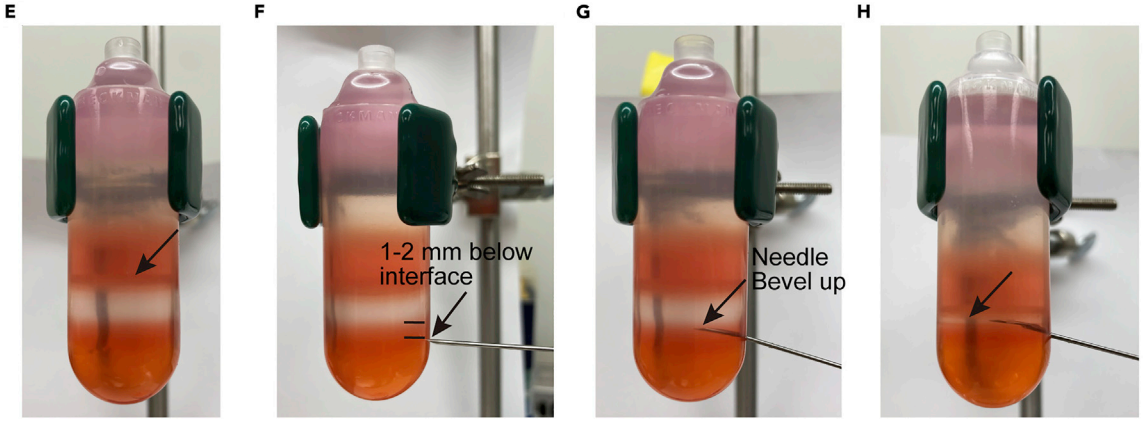

E
F
1-2 mm below interface
G
Needle Bevel up
H

Figure 4. Preparation of adeno-associated virus (AAV)

(A) Schematic diagram illustrating the transfection of AAV plasmids and AAV purification process.

(B) Preparation of iodixanol solution.

(C) Preparation of multi-layered iodixanol density gradients in OptiSeal ultracentrifuge tube.

(D) The supernatant containing the AAV is loaded onto the top layer of the iodixanol gradient and sealed tube with a cap.

(E) After ultracentrifugation, the protein-rich region (arrow) is shown at the interface of 25% and 40% iodixanol solutions.

(F and G) Insertion of the needle with the bevel up (G) below approximately 1–2 mm of the 40%–60% iodixanol interface (F).

(H) Collecting viruses should be stopped before the needle reaches the protein-rich interface as indicated by the arrow to prevent possible contamination.

a. Maintain the HEK293T cells in a 150 mm cell culture dish with 25 mL of DMEM/F-12 supplemented with 10% FBS and 1× penicillin-streptomycin at 37°C in a 5% CO_2 humidified incubator.
b. When HEK293T cells (two 150 mm cell culture dishes) reach around 80% confluence, aspirate the media and wash cells once with 20 mL of PBS to remove the residual media in the plates.
c. Detach the cells with 2 mL of Trypsin-EDTA for 2 min at 37°C.
d. Neutralize the enzymatic activity of trypsin by adding 10 mL of pre-warmed HEK293T cell culture media and suspend the cells by gentle pipetting using a 1 mL pipette.
e. Transfer the suspended HEK293T cells (from the two dishes) to a 50 mL tube.
f. Centrifuge the tube at 300 × *g* for 3 min at RT (20°C–22°C) and discard the supernatant.
g. Resuspend the cell pellet with 29 mL of pre-warmed HEK293T cell culture media.
h. Add the suspended HEK293T cells (approximately 5 mL each) dropwise into six 150 mm cell culture dishes containing 15 mL of HEK293T cell culture media.

Note: Immediately after applying the suspended HEK293T cells dropwise into each dish, spread the cells evenly on the plate surface by gently shaking the plates.

3. Transfection with plasmids into HEK293T cells for packaging of AAV.
   a. Prepare three plasmids required for packaging AAVs as below.
      i. AAV expression plasmids: pAAV-*Ef1α-CreN-InteinN* and pAAV-*Ef1α-InteinC-CreC*.
      ii. AAV-specific serotype plasmids: AAV2/8 (AAV8) and AAV-retro helper plasmids.
      iii. pAd-deltaF6 plasmid (an AAV helper plasmid that carries the genes required to drive AAV replication).

   Note: AAV2/8 (AAV8) serotype, pAAV-*Ef1α-CreN-InteinN*, and pAd-deltaF6 helper plasmids are co-transfected into HEK293T cells to package AAV8-*Ef1α-CreN-InteinN*. AAV8 serotype mediates the gene expression anterogradely from cell bodies of neurons at injected sites to their axon terminals. It is reported that AAV8 does not exhibit transsynaptic transport to postsynaptic neurons (Zingg et al., 2017). AAV-retro helper, pAAV-*Ef1α-InteinC-CreC*, and pAd-deltaF6 helper plasmids are co-transfected into HEK293T cells to package AAV-reto-*Ef1α-InteinC-CreC*. AAV-retro serotype is known to transport retrograde from axon terminals to cell bodies (Tervo et al., 2016).

   b. Twenty-four hours after seeding HEK293T cells, prepare a transfection mixture to transfect the HEK293T cells in six 150 mm cell culture dishes.
      i. Mix 90 μg of pAD-deltaF6 plasmid, 45 μg of AAV-specific serotype plasmid (AAV2/8 or AAV-retro helper plasmid), and 45 μg of AAV expression plasmid (pAAV-*Ef1α-CreN-InteinN* or pAAV- *Ef1α-InteinC-CreC*) in Opti-MEM (6 mL total volume).
      ii. Separately, mix 1 mL of 0.6 mg/mL PEI solution in 5 mL Opti-MEM and mix by vortexing.
      iii. Incubate the two mixtures separately at RT (20°C–22°C) for 10 min.
      iv. Mix the two mixtures, vortex briefly.
      v. Incubate the final mixture at RT (20°C–22°C) for 20 min.
   c. After incubation, add 2 mL of the transfection mixture dropwise to each 150 mm cell culture dish containing 18 mL of HEK293T cell culture media, and gently shake the plates.

d. 6–12 h later, aspirate the media transfection and add pre-warmed 22 mL of fresh HEK293T cell culture media.

4. Collection and lysis of transfected HEK293T cells.
   a. Seventy-two hours after transfection, scrape the transfected HEK293T cells (including cell culture media) off the 150 mm cell culture dish using a cell lifter.
   b. Transfer the cells and media from two transfected 150 mm cell culture dishes to a 50 mL tube using a 1 mL pipette.
   c. Rinse the cell culture dish with 3 mL PBS and transfer it to the same 50 mL tube.
   d. Centrifuge at 450 × *g* for 5 min at RT (20°C–22°C).
   e. Discard the supernatant by aspiration with a pasture pipette.
   f. Repeat the collection step (4a–4e) two more times to collect all the cells from the six 150 mm cell culture dishes (two dishes at a time). The same tube can be used for pelleting cells from all the dishes.

Pause point: The collected cell pellet can be stored at −80°C for later AAV purification.

Note: Before disposing of the supernatant, the collected supernatant in the flask should be treated with 10% bleach in volume. Used culture dishes should be washed with 10% bleach solution before being discarded.

   g. Prepare a dry ice/ethanol mixture bath and warm up the water bath to 37°C.
   h. Resuspend the transfected cell pellet with 4 mL cell lysis buffer.
   i. Freeze the pellet in the dry ice/ethanol mixture bath for 10 min, then thaw in a 37°C water bath for 10 min. Occasionally, shake the cell suspension thoroughly to lyse the cells and release the AAV particles.
   j. Repeat the freeze-thaw cycles four times.

Pause point: Before the fourth thawing, the frozen cell suspension can be stored at −80°C.

Note: The frozen cell suspension stored at −80°C should be thawed in a 37°C water bath for 10 min to proceed to the next step.

   k. Add Benzonase to a final concentration of 50 U/mL and vortex briefly.
   l. Incubate the tube in a 37°C water bath for 30 min to degrade DNA in the cell lysate.
   m. Centrifuge at 3,500 × *g* for 30 min at 4°C.
   n. Transfer supernatant containing AAV particles to a new 15 mL tube. The volume of the supernatant is approximately 5 mL.

Note: The supernatant can be stored at 4°C during the preparation of the iodixanol gradient.

5. Purification of AAV.

Note: Purification of AAV follows Zolotukhin and Grieger's method (Grieger et al., 2006; Zolotukhin et al., 1999) with some modifications.

   a. Make 15%, 25%, 40%, and 60% iodixanol solutions of the desired volume (Figure 4B) (see materials and equipment).

Note: Prepare multi-layered iodixanol density gradient tubes during Benzonase treatment (steps 4k–4m).

   b. Prepare two 32 mL Beckman OptiSeal ultracentrifuge tubes to purify AAV8-*CreN-InteinN* and AAV-retro-*InteinC-CreC*.

c. Add 5 mL of 60% iodixanol solution to the bottom of each tube using a 10 mL syringe with a 19-gauge needle.
d. Overlay 5 mL of 40% iodixanol solution on top of the 60% iodixanol layer using the same needle and syringe. Gently load the solution along the wall to avoid disturbing the gradient.
e. Continue the same way with 6 mL of 25% iodixanol solution and 9 mL of 15% iodixanol solution, respectively (Figure 4C).

⚠ CRITICAL: Take care to avoid bubbles forming inside the syringe. Tap the syringe to remove bubbles. The gradient can be disturbed if air bubbles are loaded into the OptiSeal ultracentrifuge tube.

f. Add 5 mL of the supernatant containing AAV particles collected in step 4n to the iodixanol gradient tube by dripping slowly along the wall onto the top layer of the gradient.
g. Fill up the iodixanol gradient tube with cell lysis buffer until the solution surface reaches the hole-neck of the tube.
h. Balance all the iodixanol gradient tubes with cell lysis buffer to make them identical in weight, and then seal the tubes with black caps (Figure 4D).

⚠ CRITICAL: When inserting the black cap into the hole, ensure that no air remains in the tube to prevent from collapsing.

i. Centrifuge at 462,000 × *g* (67,000 rpm) in Beckman Type 70 Ti rotor for 1 h at 18°C.

Note: After ultracentrifugation, the 40% iodixanol layer contains AAV particles. Cell debris and proteins are in the 25%–40% iodixanol interface (Figure 4E).

j. Carefully carry the rotor, remove the tubes from the rotor with sterilized forceps, and place the tube on the holding stand in the biosafety cabinet.
k. Prepare a 10 mL syringe with a 19-gauge needle.
l. Remove the black cap before puncturing the tube.
m. Carefully puncture the tube below approximately 1–2 mm of the 40%–60% interface (Figure 4F) with a 19-gauge needle with the bevel up (Figure 4G).
n. Withdraw 3–4 mL of the AAV-containing 40% iodixanol layer from the bottom of the layer (Figure 4H).

⚠ CRITICAL: We recommend collecting < 80% of the layer to avoid contamination with cell debris and cytotoxic proteins at the white-band interface between 25% and 40% iodixanol layers (Figures 4E and 4H).

6. Concentration of AAV.
a. Moisten the filter membrane of Amicon™ Ultra-15 Centrifugal Filter Units (100 kDa MWCO) by adding 4 mL of PBS followed by centrifugation at 4,500 × *g* for 5 min at 4°C.
b. Discard the flow-through.
c. Add the collected AAV fraction into the pre-wet Amicon™ Ultra-15 Centrifugal Filter Units.
d. Centrifuge at 4,500 × *g* for 45 min at 4°C.

Note: Ensure that the flat surface of the filter membrane is perpendicular to the direction of the fluid flow to improve the filtration efficiency.

e. Discard the flow-through and add 4 mL of pre-cooled PBS to the top of the filter.
f. Centrifuge at 4,500 × *g* for 45 min at 4°C.
g. Repeat steps 6d and 6e until the AAV solution becomes clear and the volume is approximately 50–100 μL. Usually, four times centrifugations are required.

h. Collect the AAV solution from the filter and transfer the AAV solution to a 1.5 mL tube.
i. Centrifuge at 13,000 × *g* for 20 min at 4°C to remove possible protein debris remaining in the concentrated AAV solution.
j. Transfer the supernatant containing AAV particles (approximately 45–95 μL) to a 1.5 mL tube.
k. Aliquot the AAV solution into 10 μL and store it at −80°C for long-term storage.

Note: Take a 2 μL AAV solution for AAV titration.

7. Titration of AAV using real-time qPCR.
   a. Prepare a standard curve with AAV plasmid containing the WPRE element. Make six serial dilutions of standard plasmid from 1×10^{11} to 1×10^{6} copies/mL through 10-fold dilution.
   b. Dilute the AAV solution by adding 2 μL of AAV solution to 198 μL of PBS.
   c. Add 2 μL of each diluted AAV solution and standard plasmid into 50 μL of PCR alkaline digestion buffer.
   d. Denature AAV samples and standard plasmid at 100°C for 10 min.
   e. Add 50 μL of neutralization buffer and mix by vortexing.
   f. Prepare 0.2 mL 8-Tube PCR strips.
   g. Prepare the reaction mixture to perform real-time qPCR using SYBR™ Master Mix.

PCR reaction master mix

Reagent	Amount
2× SYBR™ Master Mix	10 μL
Primer mixture (10 μM each)	1 μL
PCR template (AAV samples or standard plasmids)	3 μL
ddH_2O	6 μL
Total	20 μL

Note: Primers for qPCR:

WPRE-F: 5′-TGGCGTGGTGTGCACTGT-3′
WPRE-R: 5′-AGGGACGTAGCAGAAGGACG-3′

   h. Perform the real-time qPCR under the following PCR cycling condition using the IQ5 Real-Time PCR machine (Bio-Rad).

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	5 min	1
Denaturation	95°C	10 s	40 cycles
Annealing	60°C	10 s	
Extension	72°C	20 s	
Melting	From 60°C to 95°C	5 s	Increment 0.5°C
Hold	4°C	forever	

   i. Analyze data using iQ5 optical system software. Determine the titration of AAV samples as viral genome copies per mL. Multiply the titration of the AAV stock by 100 because the AAV sample is diluted to 1:100 ratio (from step 7b). See troubleshooting 1.

⚠ CRITICAL: Usually, virus titers are between 1×10^{13} and 5×10^{13} copies/mL. Virus titers should be high enough ($> 1 \times 10^{13}$) to express the genes with small volumes (tens to hundreds of nanoliters) to avoid any mechanical damage due to large volume infusion into the brain.

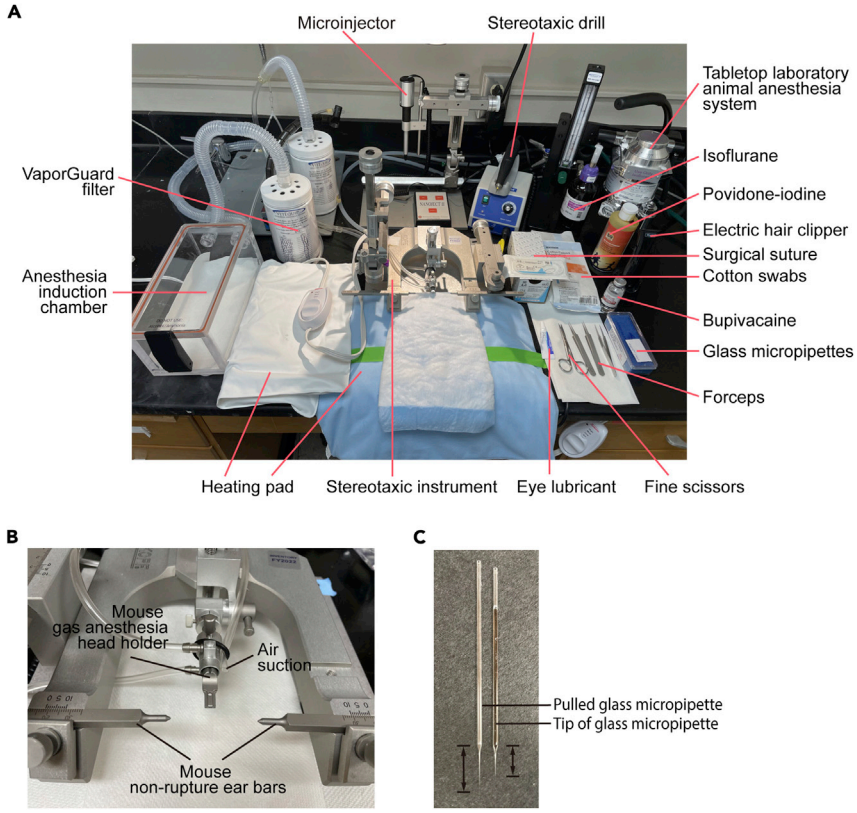


Figure 5. Preparation of surgical and stereotaxic instrument

(A) Surgery area is equipped with a stereotaxic frame, isoflurane vaporizer, stereotaxic, high-speed stereotaxic drill, microinjector, and surgical instruments such as forceps, fine scissors, and suture; surgical microscope not shown.

(B) Detailed picture of mouse gas anesthesia head holder connected with isoflurane vaporizer and air suction.

(C) Glass micropipettes for virus injection. A long and sharp-tipped glass micropipette is produced using a flaming/brown micropipette puller (left). The end of the tip is cut with scissors before use (right).

Part 3. Preparation of surgical area and injection station

Timing: 1 h

This section describes how to prepare the surgical area, surgical supplies, stereotaxic instruments, and glass micropipettes.

Note: All surgical procedures should follow the approved Institutional Animal Care and Use Committee (IACUC) protocol.

8. Using an autoclave, sterilize surgical tools and supplies, including forceps, fine scissors, and cotton swabs. Disinfect the surgical area with sporicidal disinfectant (Figure 5A).
9. Assemble the stereotaxic instrument and connect the isoflurane vaporizer to the mouse gas anesthesia head holder (Model 923-B, KOPF).
   a. Attach a vacuum tubing (air suction) to the mouse gas anesthesia head holder to evacuate any leaked isoflurane from the gas anesthesia head holder.
   b. Place a high-speed stereotaxic drill and a microinjector beside the stereotaxic instrument for easy access during surgery (Figures 5A and 5B).
10. Turn on the heating pad and set the temperature to around 37°C to ensure that the mouse maintains a constant body temperature during surgery and AAV injections.

11. Prepare the glass micropipettes (10 cm in length, 1.2 mm in outer diameter, 0.94 mm in inner diameter) for viral injections.
    a. Pull borosilicate glass capillary using a micropipette puller (Model P-97, Sutter Instruments).

Note: The parameter of the micropipette puller is as follows: TEMP 480; PULL 50; VEL 50; TIME 8.

    b. Cut the pipette tip with a scissor (desired outer diameter of pipette tip: 12–14 μm) (Figure 5C).

Note: Since the parameters for pulling micropipettes depend on the types of heating filament in the puller and the glass capillary, the parameters should be optimized if a heating filament or type of pipette is different.

Part 4. Viral injection for PFC-BLA circuit-selective genetic manipulation

Timing: 4 h

This section describes how to anesthetize the mouse and immobilize the mouse head in a stereotaxic instrument. Also, it explains how to puncture the skulls and inject the AAV8-*CreN-InteinN* and AAV-retro-*InteinC-CreC* into the PFC and BLA, respectively.

Note: To visualize the neural circuits manipulated by our split-Cre reconstitution system, we used *Ai-14* Cre-reporter mice. Animal surgeries were performed as follows, according to our previous reports (Kim et al., 2015, 2020, 2022).

12. Induction of anesthesia and fixation of mouse head in stereotaxic instruments.
    a. Place the mouse in a sealed anesthesia induction chamber supplied with isoflurane gas at 2%. This induction takes around 1–2 min.
    b. Take out the anesthetized mouse from the chamber and shave the hair of the mouse head using an electric hair clipper/trimmer. When the mouse awakens from anesthesia during shaving, place it back in the anesthesia induction chamber.
    c. Fix the head in the stereotaxic frame with two ear bars and a nose clamp equipped with a gas anesthesia head holder (Figure 6A).
    d. Supply 2% of isoflurane gas to the anesthesia head holder.
    e. Ensure that the mouse is in deep anesthesia by monitoring the reflex from the toe-pinch. If the reflex is absent, the isoflurane gas can be reduced to 1%. During surgery, the depth of anesthesia should be monitored by periodical toe-pinches.

⚠ CRITICAL: The mouse head should be fixed horizontally to the base plate and vertically to the ear bars without any medial-to-lateral or front-back movements.

    f. Apply eye lubricant to protect eyes from drying.
13. Determination of drilling points in the prefrontal cortex (PFC) and basolateral amygdala (BLA).
    a. Wipe the mouse head with 70% ethanol to remove any remaining hairs on the skin after shaving.
    b. Disinfect the scalp with 10% povidone-iodine.
    c. Make a scalp incision (15 mm) using a fine scissor (Figure 6B).
    d. Apply 2–3 drops of 0.5% bupivacaine topically on the incision area to reduce pain.
    e. Clean the exposed skull with cotton swabs dipped in sterilized PBS and then wipe with dry cotton swabs to ensure that bregma and lambda are clearly visible (Figure 6B).

Note: The bregma is the cross point of the coronal and sagittal sutures of skull and the lambda is the cross point of the lambdoid and sagittal sutures. Both the AP axis and ML

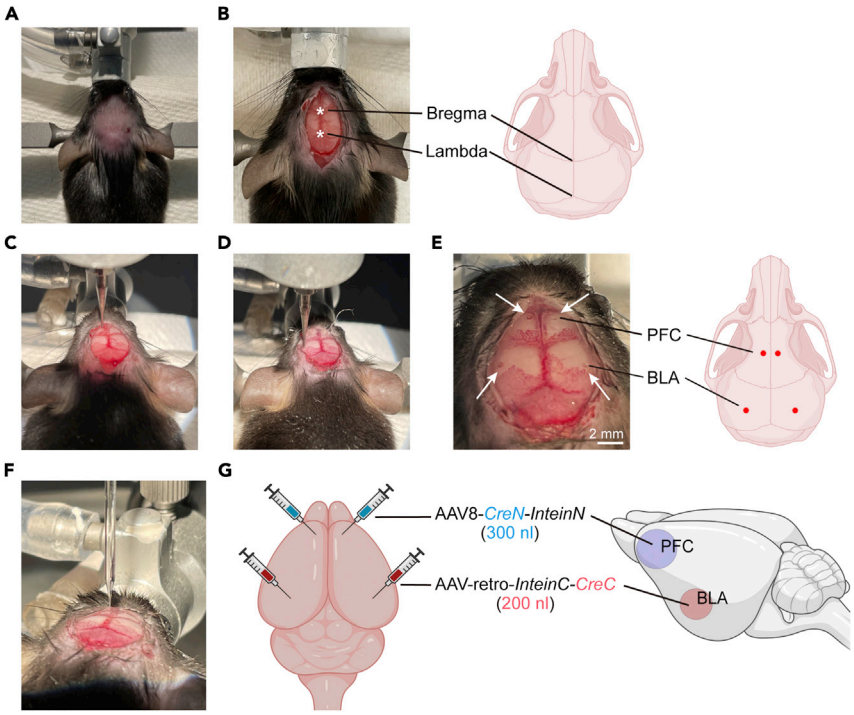


Figure 6. Injection of AAV8-*CreN-inteinN* and AAV-retro-*InteinC-CreC* into PFC and BLA, respectively

(A) Fixation of a mouse head with ear bars and gas anesthesia head holder.

(B) The bregma and lambda (asterisk) for generating the coordination of target brain regions.

(C–E) The small holes (arrows) are drilled in the skull over the PFC (C) and BLA (D) using a high-speed stereotaxic drill. The holes are made for both hemispheres to allow the AAV to be injected bilaterally into the PFC and BLA (E). Scale bar, 2 mm (E).

(F) Injection of AAV8-*CreN-InteinN* into PFC using a glass micropipette.

(G) Schematic diagram of AAV8-*CreN-InteinN* and AAV-retro-*InteinC-CreC* injections for manipulation of PFC-BLA neural circuit.

axis of the skull must be horizontal to the plate of the stereotaxic instruments to generate accurate coordinates of anatomical brain locations. The AP, ML, and DV refer to the anterior-posterior, medial-lateral, and dorsal-ventral axis, respectively.

f. Adjust the inclination of the mouse head so that the bregma and lambda are on the same horizontal plane. The difference in DV coordinates between bregma and lambda must be less than 0.05 mm.
g. Set the coordinates of the bregma as the origin of coordinates.
h. Connect a high-speed stereotaxic drill with a round 0.5 mm burr to a stereotaxic manipulator.
i. Rotate the AP and ML modulators to position the tip of the burr to move above the left PFC with the following coordinates (AP: +2.5 mm; ML: +0.8 mm).
j. Lower the tip of the burr by rotating the DV modulator slowly under a surgical microscope until the tip reaches the skull surface.
k. Carefully drill through the skull bone by lowering the burr slowly under a surgical microscope. Drill the target skull point until the dura mater beneath the skull is visible (Figure 6C). Dura mater is often punctured during drilling. If not, tear the dura mater with a 30G needle tip. Be careful not to injure the brain parenchyma. See troubleshooting 2.

⚠ CRITICAL: Occasionally, bleeding occurs while puncturing the skull. In this case, stop the bleeding by pressing the hole with a small cotton swab dipped in sterilized cold PBS. Bony

powder and excess blood should be cleaned. Cover the hole with sterilized PBS-soaked cotton to keep the brain surface moist before viral injection.

l. Reposition the bur tip to the bregma.
m. Drill holes in the skull of the right PFC and bilateral BLA in the same way as the steps 13i–13k (Figures 6D and 6E). We recommend the PFC coordinates as [AP: +2.5 mm, ML: ±0.8 mm] and BLA coordinates as [AP: -1.8 mm, ML: ±3.3 mm] in male mice aged 8 weeks.

Note: The PFC and BLA coordinates in male mice were modified and optimized based on the C57BL/J6 mouse brain atlas of Allen Institute.

14. Injections of AAV8-*CreN-InteinN* and AAV-retro-*InteinC-CreC* into the PFC and BLA, respectively.
    a. Connect a Nanoject II microinjector to a stereotaxic manipulator.
    b. Cut the tip of the glass micropipette with fine scissors to obtain a long and sharp tip.

⚠ CRITICAL: The sharp tip of the glass micropipette should be long enough not to damage the brain surface due to being pressed by the unpulled portion (shoulder) of the glass micropipette when the tip reaches the BLA coordinates.

    c. Fill the glass micropipette with mineral oil using a 28-gauge microfil flexible needle. When filling with mineral oil, remove any air bubbles in the glass micropipette. Air bubbles can impede the flow of virus solution during the injection.
    d. Mount a filled glass micropipette into the microinjector.
    e. Thaw the AAV solutions on ice.

⚠ CRITICAL: As transduction efficiency decreases by 10% each after the second and third freeze-thaw cycle, avoid multiple freeze-thaw cycles of AAV to prevent a decrease in the transduction efficiency of AAV (Howard and Harvey, 2017). If you need to use the same AAV within a week, store the remaining AAV at 4°C.

Note: Materials contaminated with AAV, such as pipette tips and glass micropipettes, are disposed of in a waste bottle containing 10% bleach.

    f. Place a piece of parafilm (~ 1 cm^2) onto the mouse head in the stereotaxic frame.
    g. Transfer a drop (1–2 μL) of AAV8-*CreN-InteinN* virus solution onto the parafilm to make it easy to load the virus into the mineral oil-filled glass micropipette.

⚠ CRITICAL: Total amount of AAV should be more than the sum of the allocations for bilateral brain injection plus the extra amount. Note that 300 nl of AAV8-*CreN-InteinN* (5.1×10^{13} copies/mL) and 200 nl of AAV-retro-*InteinC-CreC* (1.0×10^{13} copies/mL) are injected into the PFC and BLA in each hemisphere, respectively. A new glass micropipette must be used for each virus. However, the same glass micropipette can be used to inject the same AAV into bilateral brain regions.

    h. Move the glass micropipette above the drop of AAV8-*CreN-InteinN* on parafilm. Under a surgical microscope, rotate the DV modulator to position the tip of the glass micropipette within the droplet of AAV8-*CreN-InteinN*.
    i. Load the AAV8-*CreN-InteinN* slowly by pressing a Fill button on Nanoject II. Avoid air bubbles forming in the glass micropipette.
    j. Discard the parafilm into the waste bottle containing 10% bleach solution.
    k. Remove the PBS-soaked cotton used for covering the left drilled hole.

l. Move the glass micropipette above the drilled hole of the left PFC (AP: +2.5 mm; ML: +0.8 mm) and lower its tip by rotating the DV modulator slowly until the tip is close to the surface of the brain. Set the surface as a reference point for the DV coordinate.
m. Penetrate the brain by lowering the tip until it reaches -1.5 mm depth from the surface. See troubleshooting 3.
n. Leave the glass micropipette in place for at least 10 min before injection.
o. Inject 300 nl of AAV8-*CreN-InteinN* into the PFC at a flow rate of 15–20 nl/min while slowly rotating the DV modulator until the tip reaches -0.5 mm from the brain surface (Figure 6F). See troubleshooting 4.
p. After injection, place the glass micropipette in the end position (DV: -0.5 mm) for an additional 5 min to allow viral diffusion and to prevent backflow of the virus.
q. Retract the glass micropipette slowly until the tip completely leaves the skull.
r. Move the tip of the glass micropipette filled with the same virus above the right PFC with the coordinates AP: +2.5 mm and ML: -0.8 mm by rotating the AP and ML modulators.
s. Inject the 300 nl of AAV8-*CreN-InteinN* into the right PFC the same way as above.
t. Replace with new mineral oil-filled glass micropipette and load with AAV-retro-*InteinC-CreC* for injection into BLA.
u. Move the glass micropipette above the drilled hole of BLA (AP: -1.8 mm; ML: ±3.3 mm) and lower its tip slowly until the tip is close to the brain surface.
v. Penetrate the brain by lowering the tip until it reaches -4.3 mm depth from the brain surface.
w. As described in steps 14n–14q, inject 200 nl of AAV-retro-*InteinC-CreC* into each BLA (Figure 6G). The end position for BLA injection is -4.0 mm from the brain surface.

⚠ CRITICAL: An injection volume of less than 300 nl (titer: 5.1×10^{13} copies/mL) into the cell body region (PFC in this case) is recommended because virus injections of more than 300 nl induce transneuronal transport by an unknown mechanism.

x. Close the wound with a medical-grade surgical suture. Apply povidone-iodine to the suture site to prevent possible infection in the surgical wound.
y. Return the mouse to its home cage which is placed on a heating pad until it awakens and recovers from anesthesia.
z. Monitor the health status of the mouse and administer carprofen at a dose of 5 mg/kg *via* IP after surgery.

15. Allow the virus to express and reconstitute the split-Cre in the PFC-BLA neural circuit for at least 2 weeks. See troubleshooting 5.

Part 5. Imaging of PFC-BLA circuit-selective genetic manipulation

Timing: 4 days

This section describes perfusion, brain section, and imaging methods to visualize the PFC-BLA neural circuit.

Note: To image the PFC-BLA circuit manipulated by our split-Cre reconstitution system in *Ai-14* Cre-reporter mice, we conducted a circuit tracing, according to our previous reports (Kim et al., 2015, 2020, 2022).

16. Perfusion and fixation of mice two weeks after the AAV infection.
    a. Deeply anesthetize the mouse with isoflurane in an anesthesia induction chamber.
    b. Place the mouse on a perfusion plate and open the thoracic cavity to expose the heart using fine scissors.
    c. Insert the 25 gauge blunt needle into the left ventricle and make a small incision in the right atrium using fine scissors.

d. Perfuse 50–100 mL of ice-cold PBS containing 25 U/mL heparin (5 mL/min flow rate) using a peristaltic pump.
e. Perfuse 50–100 mL of ice-cold 4% PFA (5 mL/min flow rate).
f. Extract the brain and immerse it in 4% PFA at 4°C overnight (16–24 h).
g. After fixation, replace 4% PFA with 30% sucrose in PBS and allow the brain to equilibrate completely at 4°C for 2 days.

17. Sectioning of brain tissue.
    a. Place the brain tissue on the petri dish and cut the brain into the right and left hemispheres along the midline.
    b. For coronal sections, trim off the brain stem region so that the hemisphere lies flat.
    c. Place a drop of OCT compound on the chilled cryostat chuck and mount the brain hemisphere on the chuck. Ensure that the olfactory bulb of the brain faces upward.
    d. Carefully pour OCT compound on top of the brain hemisphere and return the cryostat chuck to the cryostat.
    e. Allow the brain hemisphere to freeze in the cryostat for at least 30 min.
    f. Install the chuck with the frozen brain hemisphere to the chuck holder and tighten the screw.
    g. Cut the brain hemisphere into 50 μm coronal sections and collect the sections of the PFC and BLA regions.
18. DAPI staining.
    a. Place the sections containing the PFC and BLA regions into 12 well plate containing 2 mL of 0.2% TritonX-100 in PBS per well.
    b. Place them on an orbital shaker for 15 min at RT (20°C–22°C).
    c. Stain nuclei of sections with 1 μg/mL DAPI in 2 mL of 0.2% TritonX-100 in PBS for 15 min at RT (20°C–22°C) with shaking.
    d. Wash the sections three times with 2 mL of 0.2% TritonX-100 in PBS for 5 min each.
    e. Place the sections on a slide glass and coverslip with Prolong™ glass antifade mountant.
19. Take images of the sections by tile scan imaging function using a confocal microscope (LSM 710) under the control of Zen software (Zeiss).

EXPECTED OUTCOMES

Two weeks after viral injection into *Ai-14* Cre-reporter mouse, transgenes of AAV8-*CreN-InteinN* and AAV-retro-*InteinC-CreC* will express and exclusively reconstitute within the PFC neurons that project to BLA, marking those neurons with Cre-reporting tdTomato expression (Figures 7A and 7B). This circuit-exclusive tdTomato expression can be verified by serial coronal sections from the PFC to BLA using a confocal microscope (Figure 7C). Our data show that neuronal cell bodies in the prelimbic (PL) and medial orbital (MO) cortices of the PFC express tdTomato (Figure 7D); while axon fibers pass through the medial part of the striatum (Figures 7E and 7F), and terminate in BLA (Figure 7G). These results indicate that our circuit-selective split-Intein mediated split-Cre method induces exclusive expression of Cre within the unidirectional circuit from the PFC to BLA.

The suggested combination of AAV plasmids and AAV serotype plasmids is critical as our control experiment shows that:

Injection of AAV8-*CreN-InteinN* and AAV-retro-*CreN-InteinN* into the PFC and BLA, respectively, results in no expression of tdTomato, confirming that split CreN itself has no enzymatic activity (Figures 8A–8C).

Injection of AAV9-Cre (300 nl, 2.7×10^{13} copies/mL), which induces conventional region-specific Cre expression, into the PFC results in complex axon fibers branching in various brain regions. This data suggests that our split-Intein mediated split-Cre method has a high circuit selectivity (Figures 8D–8F).

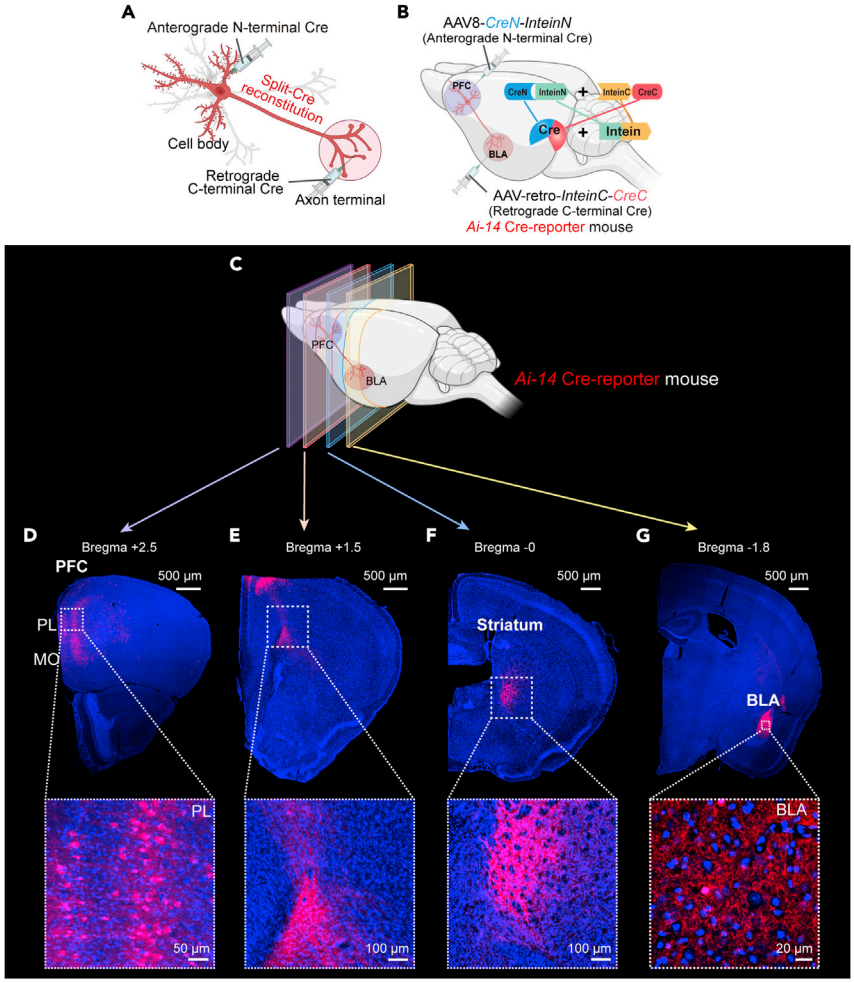


Figure 7. The tdTomato, driven by reconstituted Cre, is expressed specifically in PFC-BLA neural circuit in *Ai-14* Cre reporter mice

(A) Schematic of circuit-selective split-Cre reconstitution in a circuit neuron.

(B) Schematic of *in vivo* circuit-selective Cre expression in PFC neurons projecting to BLA.

(C) Coronal slices for representative images in (D)–(G).

(D) The tdTomato-expressing cell bodies are displayed in PL and MO regions of the PFC. Scale bar, 500 μm (upper) and 50 μm (lower).

(E and F) Axonal fibers pass through the medial part of the striatum. Scale bar, 500 μm (upper) and 100 μm (lower).

(G) Axonal fibers terminate in the BLA. Few tdTomato-expressing cell bodies were detected. Scale bar, 500 μm (upper) and 20 μm (lower).

Injection of AAV9-*CreN-InteinN* (150 nl, 5.1 × 10^{13} copies/mL) and AAV-retro-*InteinC-CreC* (50 nl, 1.0 × 10^{13} copies/mL) into the PFC and BLA, respectively, results in additional expression of tdTomato in the substantial amount of cell bodies in the BLA. This indicates that Cre is expressed in a subset of recipient neurons in the BLA due to transsynaptic transports of AAV9 (Zingg et al., 2017) (Figures 8G–8I).

Therefore, the split-Intein-mediated split-Cre method with anterograde (AAV8) and retrograde (AAV-retro) transports enable robust and highly specific unidirectional neural circuit-selective Cre expression. This method can be utilized for circuit-selective engineering of multiple genes with high specificity, such as mutating specific genes exclusively in PFC-BLA circuit neurons while simultaneously expressing Ca^{2+} sensor (*GCaMP*) or channelrhodopsin 2 (*ChR2*) in the same neurons (Kim et al., 2022).

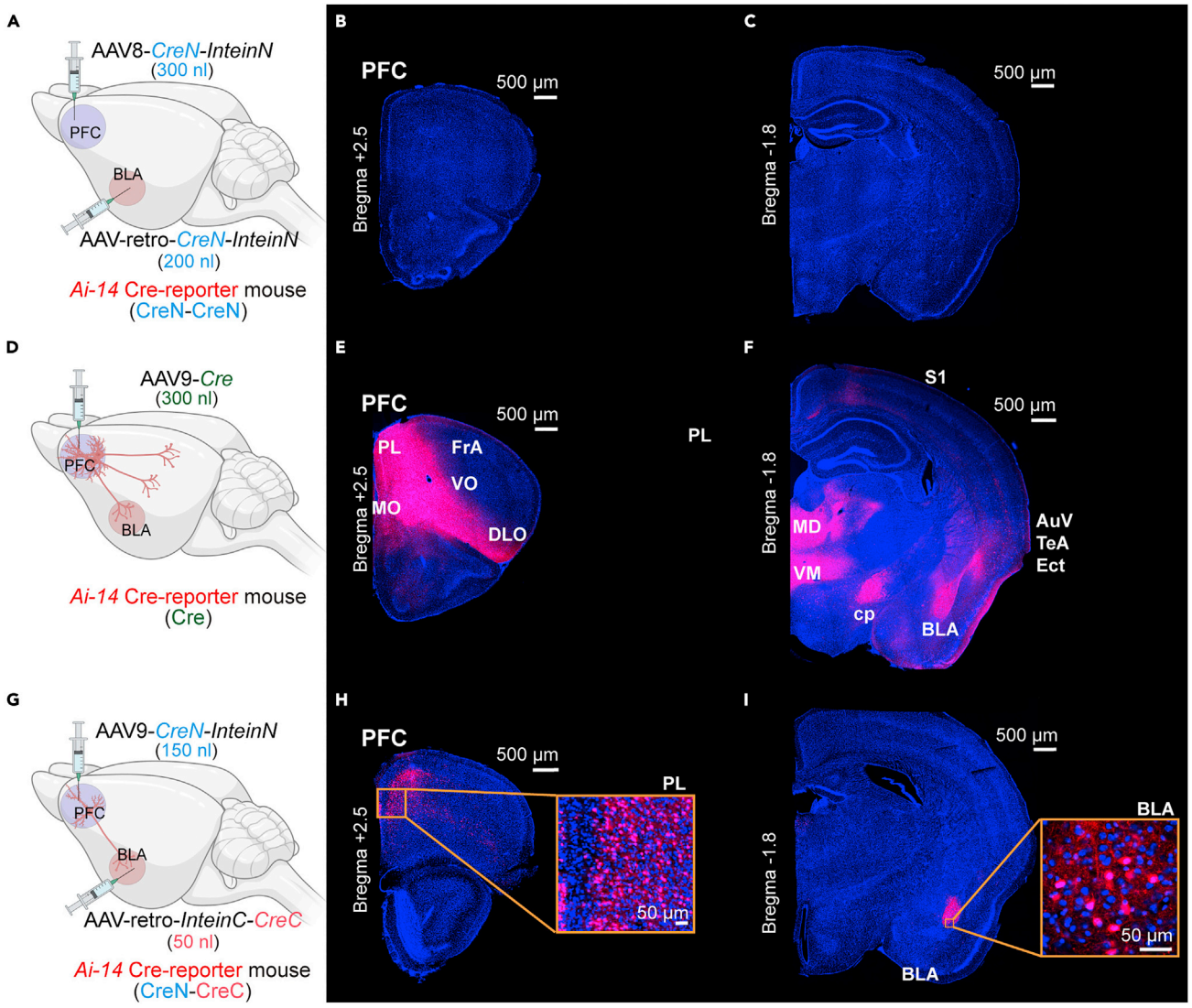


Figure 8. Validation of circuit-selective Cre expression system

(A–C) Expression of only N-terminal Cre. (A) AAV8-*CreN-InteinN* and AAV-retro-*CreN-InteinN* are injected into PFC and BLA, respectively, in the Ai-14 Cre reporter mouse. (B and C) The tdTomato expression is not detected in both PFC (B) and BLA regions (C). Scale bar, 500 μm.

(D–F) Conventional Cre expression in the PFC neurons. (D) AAV9-*Cre* is injected into the PFC of the *Ai-14* Cre reporter mouse. (E) The tdTomato signals are detected in cell bodies of the PFC region. Scale bar, 500 μm. (F) The tdTomato expressing axon fibers are shown in multiple regions as well as in the BLA region. (S1: primary somatosensory cortex; MD: mediodorsal thalamic nucleus; VM: ventromedial thalamic nucleus; AuV: secondary auditory cortex; TeA: temporal association cortex; Ect: ectorhinal cortex; cp: cerebral peduncle; BLA: basolateral amygdala). Scale bar, 500 μm.

(G–I) Circuit-selective Cre expression using AAV9-*CreN-InteinN*. (G) AAV9-*CreN-InteinN* and AAV-retro-*InteinC-CreC* are injected into PFC and BLA of *Ai-14* Cre reporter mouse, respectively. (H) The tdTomato is expressed in cell bodies of the PFC region. Scale bar, 500 μm (left) and 50 μm (right). (I) The tdTomato expressed in the BLA region is displayed in axon fibers as well as in cell bodies, indicating that transsynaptic transport is induced by the AAV9 serotype. Scale bar, 500 μm (left) and 50 μm (right).

LIMITATIONS

To manipulate specific neural circuits using this method, injections of viruses should be accomplished at accurate coordinates. Coordinates should be adjusted depending on the animals' age, gender, and weight.

In general, transgenes induced by AAV transduction are expressed in neurons within at least two weeks. However, if the expression of reconstituted Cre is weak, allow 3–4 weeks to fully express

AAV8-*CreN-InteinN* and AAV-retro-*InteinC-CreC* in the cell bodies and axon terminals of the projection neurons. Alternatively, use AAVs with a high virus titer between 1×10^{13} and 1×10^{14} copies/mL.

Although it was reported that AAV8 does not show anterograde transsynaptic transport (Zingg et al., 2017), we observed that a large volume of high titer AAV8 injection could undergo some level of anterograde transneuronal transport. As mentioned above, we recommend using less than 300 nl (titer: 5.1×10^{13} copies/mL) of AAV8-*CreN-InteinN* virus volume. We also recommend testing and optimizing the AAV8-*CreN-InteinN* titer and volume when targeting a specific neural circuit of interest other than the PFC-BLA circuit.

TROUBLESHOOTING

Problem 1

The low titer of AAVs after AAV purification (step 7i).

Potential solution

Low titers of AAVs may be caused by unhealthy HEK293T cells, low transfection efficiency, or incomplete HEK293T cell lysis. Healthy HEK293T cells of the mycoplasma-free and low passage should be used for AAV production. Seed an appropriate number of HEK293T cells 24 h prior to transfection to ensure that the HEK293T cells are 80% confluent in a 150 mm dish when transfecting into HEK293T cells. To ensure high transfection efficiency, optimize the transfection conditions, including the amount and ratio of the three plasmids, the transfection reagent, and the incubation time after transfection. Finally, the transfected HEK293T cells should be completely lysed to obtain a high amount of AAV. Shake frequently the cell suspension in a 37°C water bath during the freeze/thaw cycle to increase cell lysis. Do not vortex the cell suspension.

Problem 2

Excessive bleeding while puncturing the brain skull and inserting a glass micropipette into the brain (step 13k).

Potential solution

Bleeding can occur when puncturing the skull located above large blood vessels and sinuses. When bleeding occurs, repeatedly clean the hole with sterilized cold PBS-soaked cotton swabs. If the bleeding does not stop, apply a hemostatic sponge (Gelfoam®) to the hole until the bleeding stops.

Problem 3

Bending of the glass micropipette tip while penetrating the brain (step 14m).

Potential solution

This commonly happens because the dura mater under the skull is a thick and strong membrane layer. If the meninges are not completely punctured during drilling a hole, the glass micropipette tip may bend or break. If necessary, ensure that the meninges have been completely removed before proceeding to injections using a sharp needle (30 Gauge).

Problem 4

The backflow of AAV solution while injecting viruses into the brain (step 14o).

Potential solution

Backflow of AAV solution can be observed during virus injection. In this case, viruses can spread to undesired regions along the micropipette track. We recommend injecting the viruses at a flow rate of 15–20 nl/min so that the viruses can be slowly absorbed into the brain parenchyma.

Problem 5

Decreased efficiency of neural circuit manipulation due to low expression of reconstituted Cre (step 15).

Potential solution

Check the virus titer of AAV8-*CreN-InteinN* and AAV-retro-*InteinC-CreC*. High titer viruses of 1×10^{13} to 1×10^{14} copies/mL are recommended. To maintain the AAV solution at a high titer, store AAV stock in small aliquots (10 μL) and avoid frequent freeze-thaw cycles. Additionally, confirm the accuracy of injection coordinates and the volume of virus injection. However, since the BLA region is small, inject *AAV-retro-IntecC-CreC* less than 200 nl to prevent unwanted infections around BLA.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Il Hwan Kim (ikim9@uthsc.edu).

Materials availability

The pAAV-*Ef1α-CreN-InteinN* and pAAV-*Ef1α-InteinC-CreC* plasmids have been deposited at Addgene with ID 187614 (https://www.addgene.org/187614/) and ID 187615 (https://www.addgene.org/187615/), respectively. The AAV-*Ef1a-Flex-GFP* plasmid is available from the lead contact upon request.

Data and code availability

This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS

Y.K., S.K., and I.H.K. designed this study. Y.K. and I.H.K. performed virus design and cloning. Y.K. performed virus purification. S.K. performed viral injections and circuit tracing. Y.K., S.K., and I.H.K. performed data analyses. This paper was written by Y.K., S.K., and I.H.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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