Protocol

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.
Protocol

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

Yong-Eun Kim,1,2,3,4 Sunwhi Kim,1,2,3 and Il Hwan Kim1,2,5,*

1Department of Anatomy and Neurobiology, University of Tennessee Health Science Center, Memphis, TN 38163, USA
2Neuroscience Institute, University of Tennessee Health Science Center, Memphis, TN 38163, USA
3These authors contributed equally
4Technical contact: ykim73@uthsc.edu
5Lead contact
*Correspondence: ykim73@uthsc.edu (Y.-E.K.), skim135@uthsc.edu (S.K.), ikim9@uthsc.edu (I.H.K.)
https://doi.org/10.1016/j.xpro.2022.101807

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.
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 $5 \times 10^5$ cells/well that contains 500 µL of DMEM/F-12 supplemented with 10% FBS and 1x penicillin-streptomycin, and then incubate at 37°C overnight (16–24 h) in a 5% CO₂ 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.
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 1x penicillin-streptomycin) to each chamber.

5. Incubate the transfected HEK293T cells at 37°C in a 5% CO2 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-E1α-CreN-inteinN, pAAV-E1α-inteinC-CreC, and AAV-E1α-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; Sbi3 | 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 |

(Continued on next page)
| 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 |
| SporCon Sporicidal Disinfectant | Decon | 4301 |
| Tris | IBI | IB70145 |
| Sodium chloride (NaCl) | Sigma-Aldrich | S9888 |
| Potassium chloride (KCl) | Sigma-Aldrich | P3911 |
| Sodium phosphate dibasic (Na2HPO4) | Sigma-Aldrich | S9763 |
| Potassium phosphate monobasic (KH2PO4) | Sigma-Aldrich | P0662 |
| Magnesium chloride (MgCl2) | 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 | | |
| WPWE-F | IDT | 5’TGG CGT GGT GTG CAC TGT-3’ |
| WPWE-R | IDT | 5’AGG GAC GTA GCA AAA 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., 2023) https://doi.org/10.1016/j.celrep.2022.110906 | 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 |
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Zen software        | ZEISS  | [https://www.zeiss.com/microscopy/en/products/software/zeiss-zen.html](https://www.zeiss.com/microscopy/en/products/software/zeiss-zen.html) |
| 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 B-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 |
| Precision Glide 19G x 1 1/2” Needle | BD | 305187 |
| Precision Glide 30G x 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 x 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 |
| IQS Real-Time PCR Complete system | Bio-Rad | IQS |

(Continued on next page)
**Materials and Equipment**

**LB Broth**

| Reagent                              | Amount |
|--------------------------------------|--------|
| LB Lennox Broth                      | 20 g   |
| double-distilled H₂O (ddH₂O)         | ~ 950 mL |
| **Total**                            | 1 L    |

Adjust the volume to 1 L with additional ddH₂O. 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 H₂O (ddH₂O)         | ~ 450 mL |
| **Total**                            | 500 mL |

Adjust the volume to 500 mL with additional ddH₂O. After autoclaving at 121 °C for 15 min, leave the LB agar solution to cool to approximately 55 °C. Add the 500 mL 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 (100X) | 1 X                | 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  |
| Na₂HPO₄                               | 10 mM               | 1.44 g |
| KH₂PO₄                               | 1.8 mM              | 0.24 g |
| double-distilled H₂O (ddH₂O)         | N/A                 | ~ 900 mL |
| **Total**                            |                     | 1 L    |

Adjust pH to 7.4 and adjust the volume to 1 L with additional ddH₂O. 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<sub>2</sub> | 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<sub>2</sub> | 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<sub>2</sub>O | ~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<sub>2</sub>O. 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<sub>2</sub>O  | N/A                | ~910 mL|
| **Total**        |                     | 1 L    |

Adjust pH to 8.5 and adjust the volume to 1 L with ddH<sub>2</sub>O. 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<sub>2</sub>O (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<sub>2</sub>O. 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  |

Prepare the fresh solution before use. The volume of iodixanol solution is for two OptiSeal tubes.

### 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|
| ddH₂O         | 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   |
| ddH₂O         | 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   |
| 1x PBS        | ~ 900 mL |
| **Total**     | 1 L    |

Add 1x 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 1x 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 |
| 1x PBS        | ~ 990 mL |
| **Total**     | 1 L    |

Adjust the volume to 1 L with 1x PBS and 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 (Ca2+ /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’-GCGCACGCCT-3’) and Kpn1 (5’-GGTACCGCGC-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.

| 30% Sucrose in PBS | Amount     |
|-------------------|------------|
| Reagent           |            |
| Sucrose           | 150 g      |
| 1x PBS            | ~ 300 mL   |
| Total             | 500 mL     |

Adjust the volume to 500 mL with 1x PBS and store at 4°C for up to 1 month.

| 0.2% TritonX-100/PBS | Amount   |
|----------------------|----------|
| Reagent              |          |
| TritonX-100          | 1 mL     |
| 1x PBS               | 499 mL   |
| Total                | 500 mL   |

Store at 4°C for up to 1 month.
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.
Protocol

A

Transfection

Collection of transfected cells

AAV purification using Iodixanol gradient

Dialysis and AAV concentration

AAV solution

AAV titration

B

Iodixanol solution

15% 25% 40% 60%

C

Cell supernatant (AAV)

15% Iodixanol

25% Iodixanol

40% Iodixanol

60% Iodixanol

D

1-2 mm below interface

Needle Bevel up

E

F

G

H
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% CO2 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 3000 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.
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 \( \times \) 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 \( \mu \)L) to a 1.5 mL tube.
k. Aliquot the AAV solution into 10 \( \mu \)L and store it at −80°C for long-term storage.

**Note:** Take a 2 \( \mu \)L 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 \( 1 \times 10^{11} \) to \( 1 \times 10^{6} \) copies/mL through 10-fold dilution.
   b. Dilute the AAV solution by adding 2 \( \mu \)L of AAV solution to 198 \( \mu \)L of PBS.
   c. Add 2 \( \mu \)L of each diluted AAV solution and standard plasmid into 50 \( \mu \)L of PCR alkaline digestion buffer.
   d. Denature AAV samples and standard plasmid at 100°C for 10 min.
   e. Add 50 \( \mu \)L 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.

Note: Primers for qPCR:

WPRE-F: 5’-TGGCGTGGTGCTGCACGTG-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 reaction master mix | Amount |
|-------------------------|--------|
| 2x SYBR™ Master Mix     | 10 \( \mu \)L |
| Primer mixture (10 \( \mu \)M each) | 1 \( \mu \)L |
| PCR template (AAV samples or standard plasmids) | 3 \( \mu \)L |
| ddH2O                   | 6 \( \mu \)L |
| Total                   | 20 \( \mu \)L |

Note: Primers for qPCR:

WPRE-F: 5’-TGGCGTGGTGCTGCACGTG-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 \( 1 \times 10^{13} \) and \( 5 \times 10^{13} \) 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.
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
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 cm²) 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 x 10¹³ copies/mL) and 200 nl of AAV-retro-InteinC-CreC (1.0 x 10¹³ 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).
Injection of AAV9-CreN-InteinN (150 nl, 5.1 x 10^{13} copies/mL) and AAV-retro-InteinC-CreC (50 nl, 1.0 x 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).
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 $1 \times 10^{13}$ and $1 \times 10^{14}$ 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: $5.1 \times 10^{13}$ 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 $1 \times 10^{13}$ to $1 \times 10^{14}$ copies/mL are recommended. To maintain the AAV solution at a high titer, store AAV stock in small aliquots ($10 \mu$L) 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 (kim9@uthsc.edu).

Materials availability
The pAAV-Ef1a-CreN-InteinN and pAAV-Ef1a-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.

ACKNOWLEDGMENTS
This work was supported by the National Institutes of Health (NIH) research grant MH117429 and NARSAD Young Investigator Grant 25163 to I.H.K. We thank Dr. Fan Wang for providing the Intein-Cre sequence and Dr. Matthew Ennis for critical reading and comments. A subset of the illustrations was created with BioRender.com.

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.

REFERENCES
Al-Allaf, F.A., Tolmachov, O.E., Zambetti, L.P., Tchetchelnitski, V., and Mehmet, H. (2013). Remarkable stability of an instability-prone lentiviral vector plasmid in Escherichia coli Stbl3. 3 Biotech 3, 61–70. https://doi.org/10.1007/s13205-012-0070-8.

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/elif.e61590.

Dimidschstein, J., Chen, Q., Tremblay, R., Rogers, S.L., Saldi, G.A., Guo, L., Xu, Q., Liu, R., Lu, C., Chu, J., et al. (2016). A viral strategy for targeting and manipulating interneurons across vertebrate species. Nat. Neurosci. 19, 1743–1749. https://doi.org/10.1038/nn.4430.

Evans, T.C., Jr., Martin, D., Kolly, R., Panne, D., Sun, L., Ghosh, I., Chen, L., Benner, J., Liu, X.Q., and Xu, M.Q. (2000). Protein trans-splicing and cyclization by a naturally split intein from the dnaE gene of Synechocystis species PCC6803. J. Biol. Chem. 275, 9091–9094. https://doi.org/10.1074/jbc.275.13.9091.

Feil, R., Brocard, J., Mascrez, B., LeMeur, M., Metzger, D., and Chambon, P. (1996). Ligand-activated site-specific recombination in mice. Proc. Natl. Acad. Sci. USA 93, 10887–10890. https://doi.org/10.1073/pnas.93.20.10887.

Gray, S.J., Foti, S.B., Schwartz, J.W., Bachaboina, L., Taylor-Blake, B., Coleman, J., Ehlers, M.D., Zylka, M.J., McCown, T.J., and Samulski, R.J. (2011). Optimizing promoters for recombinant adeno-associated virus-mediated gene expression in the peripheral and central nervous system using self-complementary vectors. Hum. Gene Ther. 22, 1143–1153. https://doi.org/10.1089/hum.2010.245.

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.

Haery, L., Deverman, B.E., Matho, K.S., Cetin, A., Woodard, K., Cepko, C., Guerin, K.I., Rego, M.A., Ersing, I., Bachle, S.M., et al. (2019). Adeno-associated virus technologies and methods for targeted neuronal manipulation. Front. Neuroanat. 13, 93. https://doi.org/10.3389/fnana.2019.00093.
Hoesche, C., Sauerwald, A., Veh, R.W., Krippel, B., and Kilimann, M.W. (1993). The 5'-flanking region of the rat synapsin I gene directs neuron-specific and developmentally regulated reporter gene expression in transgenic mice. J. Biol. Chem. 268, 26494–26502.

Howard, D.B., and Harvey, B.K. (2017). Assaying the stability and inactivation of AAV serotype 1 vectors. Hum. Gene Ther. Methods 28, 39–48. https://doi.org/10.1089/hgtb.2016.180.

Kim, I.H., Kim, N., Kim, S., Toda, K., Catavero, C.M., Courtland, J.L., Yin, H.H., and Soderling, S.H. (2003). Dysregulation of the synaptic cytoskeleton in the PFC drives neural circuit pathology, leading to social dysfunction. Cell Rep. 32, 107965. https://doi.org/10.1016/j.celrep.2020.107965.

Kim, I.H., Rossi, M.A., Aryal, D.K., Racz, B., Kim, N., Uezu, A., Wang, F., Wetsel, W.C., Weinberg, R.J., Yin, H., and Soderling, S.H. (2015). Spine pruning drives antipsychotic-sensitive locomotion via circuit control of striatal dopamine. Nat. Neurosci. 18, 883–891. https://doi.org/10.1038/nn.4015.

Kim, S., Kim, Y.E., Song, I., Uijihara, Y., Kim, N., Jiang, Y.H., Yin, H.H., Lee, T.H., and Kim, I.H. (2022). Neural circuit pathology driven by Shank3 mutation disrupts social behaviors. Cell Rep. 39, 110906. https://doi.org/10.1016/j.celrep.2022.110906.

McCown, T.J., Xiao, X., Li, J., Breese, G.R., and Samulski, R.J. (1996). Differential and persistent expression patterns of CNS gene transfer by an adeno-associated virus (AAV) vector. Brain Res. 713, 99–107. https://doi.org/10.1016/0006-8993(95)01488-8.

Nathanson, J.L., Yanagawa, Y., Obata, K., and Callaway, E.M. (2009). Preferential labeling of inhibitory and excitatory cortical neurons by endogenous tropism of adeno-associated virus (AAV) vector. Neuroscience 161, 441–450. https://doi.org/10.1016/j.neuroscience.2009.03.032.

Tervo, D.G.R., Hwang, B.Y., Viswanathan, S., Gaj, T., Lavzin, M., Ritala, K.D., Lindo, S., Michael, S., Kuleshova, E., Ogala, D., et al. (2016). A designer AAV variant permits efficient retrograde access to projection neurons. Neuron 92, 372–382. https://doi.org/10.1016/j.neuron.2016.09.021.

Wang, P., Chen, T., Sakurai, K., Han, B.X., He, Z., Feng, G., and Wang, F. (2012). Intersectional Cre driver lines generated using split- intein mediated split-Cre reconstitution. Sci. Rep. 2, 497. https://doi.org/10.1038/srep00497.

Wu, H., Hu, Z., and Liu, X.Q. (1998). Protein transsplicing by a split intein encoded in a split DnaE gene of Synechocystis sp. PCC6803. Proc. Natl. Acad. Sci. USA 95, 9226–9231. https://doi.org/10.1073/pnas.95.16.9226.

Zingg, B., Chou, X.L., Zhang, Z.G., Mesik, L., Liang, F., Tao, H.W., and Zhang, L.L. (2017). AAV-mediated anterograde transsynaptic tagging mapping corticocollicular input-defined neural pathways for defense behaviors. Neuron 93, 33–47. https://doi.org/10.1016/j.neuron.2016.11.045.

Zolotukhin, S., Byrne, B.J., Mason, E., Zolotukhin, I., Potter, M., Chesnut, K., Summerford, C., Samulski, R.J., and Muzyczka, N. (1999). Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. Gene Ther. 6, 973–985. https://doi.org/10.1038/sj.gt.3300938.