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bio-protocol www.bio-protocol.org/e4246 Bio-protocol 11(23): e4246. DOI:10.21769/BioProtoc.4246

Conditional Gene Editing in Presynaptic Extinction-ensemble Cells via the CRISPR-SaCas9 System

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[Abstract] The CRISPR-Cas9 enables efficient gene editing in various cell types, including post-mitotic neurons. However, neuronal ensembles in the same brain region can still be functionally or anatomically different, and such heterogeneity requires gene editing in specific neuronal populations. We recently developed a CRISPR-SaCas9 system-based technique. Combined with activity-dependent cell-labeling methods and anterograde/retrograde adeno-associated virus (AAV) vectors, this technique achieves function- and projection-specific gene editing in the mammalian brain. We showed that perturbing *cbp* (CREB-binding protein) in extinction-ensemble neurons among amygdala-projecting infralimbic cortex (IL) cells impaired fear extinction learning, demonstrating the high efficiency in regulation of extinction learning with CRISPR-Cas9. Here, we describe a detailed protocol of gene perturbation in presynaptic extinction-ensemble neurons in adult rats, including gRNA design, gRNA evaluation *in vitro*, stereotaxic AAV injection, and contextual fear conditioning. The high specificity and efficiency of projection- and function-specific CRISPR-SaCas9 system can be widely applied in neural circuitry studies.

Keywords: Gene editing, CRISPR-SaCas9, Infralimbic cortex, Memory, Adeno-associated virus

[Background] In a brain region, neuronal ensembles can divide into distinct subpopulations, either functionally, by their recruitment in various tasks, or anatomically, by their efferent/afferent connections. It is still challenging to decipher the logic of complex neuronal networks and molecular underpinnings of memory, which highlights the requirement for conditional gene perturbation in the heterogeneous brain with precise spatial and temporal resolution. A conditional recombination system has been broadly applied to investigate brain function with spatiotemporal accuracy, but the construction of germline-modified mutant strains can be time-consuming and labor-intensive, especially for transgenic rats.

Emerged in 2013, the CRISPR-Cas9 system induces frameshifting insertion/deletion (indel) mutations, thus allowing efficient perturbation of endogenous genes in different cell types (Cong *et al.*, 2013) and functional analysis of specific genes in the mammalian brain (Swiech *et al.*, 2015). Cre-loxP tools that allow conditional control of spatiotemporal expression of Cas9 have been established in rodents (Platt *et al.*, 2014; Bäck *et al.*, 2019), enabling precise gene manipulation in defined neuronal types, including engram cells, the putative substrate for memory storage (Liu *et al.*, 2012). Nevertheless, the restrictive

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cargo size (usually less than 4.5 kb) of the highly versatile AAV vehicle presents an obstacle for efficient packaging of the commonly used SpCas9 (*Streptococcus pyogenes*, 4.2 kb). By contrast, the Cas9 ortholog from *Staphylococcus aureus* (SaCas9, 3.2 kb) is 1 kb shorter but edits the genome with an efficiency similar to SpCas9 (Ran *et al.*, 2015). Combining SaCas9, AAV vector, and activity-dependent cell-labeling techniques, we recently developed a conditional genome perturbation in engram cells bearing memory storage or extinction, thereby impairing the remote memory or extinction learning in rats (Sun *et al.*, 2020).

As a proof-of-principle protocol, we describe here how to knockdown *cbp* in presynaptic IL extinction-ensemble neurons (Figure 1), which can be used to investigate the circuit and molecular basis of memory.

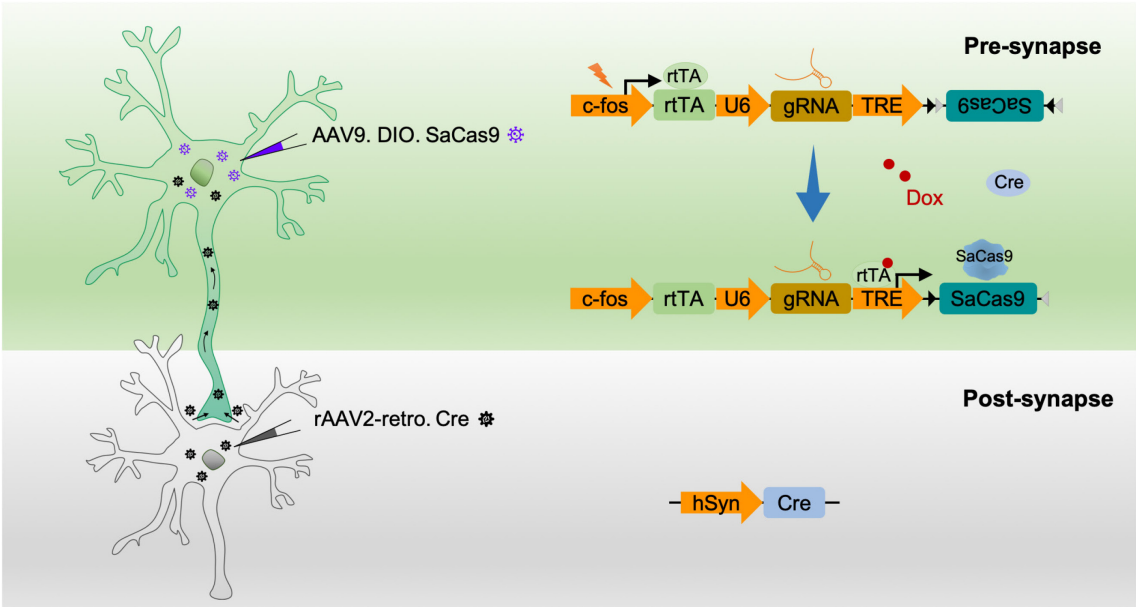

Figure 1. Schematic of conditional gene editing in the brain.

With doxycycline (Dox), training induces the expression of reverse tetracycline transactivator (rtTA), which binds to tetracycline responsive element (TRE), and drives the expression of SaCas9, thereby inducing an indel in the *cbp* locus of activated neurons (top panel). Presynaptic expression of SaCas9 is achieved by retrograde rAAV2-retro expressing the Cre recombinase (bottom panel). c-fos, the promoter of *c-fos*, an immediate early gene frequently used as a marker of neuronal activity; U6, Pol III promoter; hSyn, human synapsin 1 promoter; rtTA, reverse tetracycline transactivator; TRE, tetracycline responsive element; gRNA, guide RNA; SaCas9, Cas9 ortholog from *Staphylococcus aureus*; Dox, doxycycline; Cre, Cre recombinase; DIO, double-floxed inverted open reading frame.

Materials and Reagents

1. 0.45-μm polyvinylidene difluoride (PVDF) filter (Millipore, catalog number: SLHV033RB)
2. 1,000 μl, 200 μl, 10 μl pipette tips (Kirgen, catalog numbers: KG1333, KG1232, KG1031)

3. 6-well cell culture plate (Corning, catalog number: 3516)
4. 24-well cell culture plate (Corning, catalog number: 3527)
5. 60-mm cell culture dish (Corning, catalog number: 430166)
6. 100-mm cell culture dish (Corning, catalog number: 430167)
7. Eppendorf tubes, 1.5-ml (Eppendorf, catalog number: 0030125150)
8. 15-ml centrifuge tubes (Corning, catalog number: 430791)
9. 50-ml centrifuge tubes (Corning, catalog number: 430829)
10. Cryogenic tube (Corning, catalog number: 430659)
11. Cotton swab
12. 293T cell line (American Type Culture Collection, catalog number: CRL-3216)
13. F98 cell line (American Type Culture Collection, catalog number: CRL-2397)
14. Dulbecco's Modified Eagle Medium (DMEM; Gibco, catalog number: 11995065)
15. Opti-MEM (Gibco, catalog number: 11058021)
16. Fetal bovine serum (FBS; Gibco, catalog number: 10099141C)
17. Phosphate buffered saline (PBS; Gibco, catalog number: 10010023)
18. Penicillin-streptomycin (5,000 U/ml) (Gibco, catalog number: 15070063)
19. Trypsin (0.25%), phenol red (Gibco, catalog number: 15050065)
20. Ampicillin (Coolaber, catalog number: LG-CA0006)
21. Polybrene transfection reagent (Millipore, catalog number: TR-1003-G)
22. VigoFect (Vigorous biotechnology, catalog number: T001)
23. Tryptone (OXOID, catalog number: LP0042B)
24. Yeast extract (OXOID, catalog number: LP0021B)
25. Triton X-100 (Thermo Fisher Scientific, catalog number: BP151-100)
26. Chlorine bleach
27. Lentivirus packaging plasmid pMD2.G (Addgene, catalog number: 12259)
28. Lentivirus packaging plasmid psPAX2 (Addgene, catalog number: 12260)
29. Universal genomic DNA kit (CWBIO, catalog number: CW2298S)
30. Endofree plasmid midi kit (CWBIO, catalog number: CW2105S)
31. EnGen mutation detection kit (Kit components: Q5 Hot Start High-Fidelity 2× Master Mix, NEBuffer 2, EnGen T7 Endonuclease I, Proteinase K. New England Biolabs, catalog number: E3321S)
32. Flag antibody (Sigma-Aldrich, catalog number: F1804)
33. Alexa Fluor 594 goat anti-mouse IgG (ZSGB-BIO, catalog number: ZF-0513)
34. Isoflurane (RWD Life Science, catalog number: R510-22)
35. Doxycycline diet, 100 ppm (ReadyDietech, catalog number: RD07053006)
36. rAAV2-retro-hSyn-Cre-P2A-GFP (1.7×10^{13} v.g./ml) is purchased from OBiO Technology (Shanghai, China)

    Note: Aliquots should be stored at -80°C.
37. Customized AAV9-TRE^{3G}-DIO-SaCas9-3×flag (3.2×10^{13} v.g./ml) and AAV9-c-fos-rtTA-U6-

gRNA (1.7×10^{13} v.g./ml) are constructed and packaged from Vigene Biosciences (Jinan, China)
Note: Aliquots should be stored at -80°C.

38. Complete growth medium for 293T and F98 cells (see Recipes)

Equipment

1. Pipettes (Gilson, Pipetman classic P10, P200, P1000)
2. -80°C freezer (Haier, model: DW-86L388J)
3. Class II biosafety cabinet (ESCO, model: AC2-4S8-CN)
4. Thermal cycler (Bio-Rad, model: C1000 Touch)
5. Centrifuge with refrigeration (Eppendorf, model: 5424R)
6. CO_2 incubator (Thermo Fisher Scientific, model: Heracell 240i)
7. Confocal laser-scanning microscope (Leica, model: TCS SP8)
8. Gel electrophoresis chamber (Bio-Rad, model: Mini-Sub Cell GT Cell)
9. Small animal anesthesia machine (RWD Life Science, model: R500)
10. Digital stereotaxic instrument (RWD Life Science, model: 68026)
11. Microsyringe, 1 μl/OD: 0.55 mm (RWD Life Science, model: 79002)
12. Surgical scissors (RWD Life Science, model: S14001)
13. Hemostatic forceps (RWD Life Science, model: F22006)
14. Surgical forceps (RWD Life Science, model: F13017)
15. Medical stitches (RWD Life Science, model: F33309)
16. Cranial drill (RWD Life Science, model: 78001)
17. Drill bits (RWD Life Science, model: 78043)
18. Microsyringe pump (KD Scientific, model: Legato 130)
19. Cryostat microtome (Leica, model: 1950)
20. Fear conditioning and startle system (Panlab, Spain, model: LE116)

Software

1. CRISPR RGEN Tools (http://www.rgenome.net/cas-designer/)
2. GraphPad Prism 8 (Downloaded from https://www.graphpad.com/)
3. ImageJ software (Downloaded from NIH webpage)
4. Packwin V2.0 software (Panlab, Spain)

Procedure

A. gRNA design

1. For selection of the gRNA target, we recommend CRISPR RGEN Tools (www.rgenome.net/cas-designer/). The 21-nucleotide target sequences should target early exons (near transcriptional

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start site), or critical exons of gene function, preceding a 5′-NNGRRT protospacer adjacent motif (PAM for SaCas9) sequence. We recommend choosing targeting sequences with higher out-of-frame score (⩾ 65 is recommended) and with less predicted mismatches (⩽ 3 mismatched bases is not recommended) throughout the whole genome, to minimize the off-target effect of SaCas9. The nucleotide GC contents should be between 20% to 80%. Discard targets with more than 4 repeated thymines (TTTT) to reduce possible termination by Polymerase III. An extra “G” nucleotide should be appended to the 5′ end of the gRNA, which is required for strong expression from the U6 promoter (Table 1).

2. Lentiviral vectors (CMV-SaCas9-P2A-mCherry-U6-gRNA) expressing SaCas9 and gRNA used in this protocol are commercially available from Vigene Biosciences.

Table 1. gRNA sequences used in this protocol

	gRNA sequence targeting *cbp*	PAM	Exon	Direction
gRNA-control	GGAGACGAATAATGCGTCTCC	/	/	/
CBP-gRNA-1	CTGGCTGCCTGTTTGGGCAGG	CTGGGT	2	-
CBP-gRNA-2	AAATGTCAGCGACGAGAGCAA	GCGAAT	4	+
CBP-gRNA-3	AGCAGCTGTGGAAGCAGGAGG	TGGAGT	14	-
CBP-gRNA-4	ATTCAGTGCTTGGGAGGCAGG	GGGAGT	2	-
CBP-gRNA-5	AGTAACTCTGGCCATAGCTTA	ATGAAT	2	+

B. gRNA evaluation *in vitro*

1. Lentivirus production

   a. The day before transfection, seed approximately 500k 293T cells into a 60-mm dish with 4 ml of complete growth medium. Distribute 293T cells evenly across the dish by shifting the dish side-to-side and forward-backward.

      Note: The health of 293T cells is essential for virus packaging. Never use cells that are above 25 passages, growing unevenly, or looking morphologically abnormal.

   b. Carefully check the growth rate of 293T cells; we recommend a 40-60% confluence before lentivirus packaging. Replace the cell culture medium with fresh one (2 ml) 1 h before transfection, then incubate in CO_2 incubator at 37°C.

   c. Aseptically transfer 200 µl of Opti-MEM to a 1.5 ml tube for each dish to be transfected. Add 400 ng CMV-SaCas9-P2A-mCherry-U6-gRNA, 200 ng pMD2.G, and 300 ng psPAX2 to the medium and slowly pipet to mix.

   d. Aseptically transfer another 200 µl of Opti-MEM to a 1.5 ml tube for each dish to be transfected. Add 4 µl of VigoFect to the 200 µl of Opti-MEM, slowly pipet to mix, and equilibrate to room temperature for 5 min.

   e. Add VigoFect solution into the plasmid solution drop by drop. Pipet the transfection solution gently and avoid making bubbles, then equilibrate to room temperature for 15 min.

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f. Add the transfection solution to 293T cells drop by drop, and gently mix by shifting the dish.
g. Replace the culture medium with fresh one (4 ml) 6 h after transfection.
h. Validate mCherry expression in 293T cells with a fluorescent microscope 24 h after transfection—the transfection efficiency should be above 80%.
i. Harvest the lentivirus supernatant in a 15 ml centrifuge tube, 36 and 60 h after transfection.
j. Centrifuge the lentivirus supernatant at 3,000 rpm for 5 min to pellet debris, and collect the supernatant in a new tube.
k. Filter the lentivirus supernatant with a 0.45-μm PVDF filter, aliquot the lentivirus solution into single use vials, then store at -80°C.

Notes:

i. Comply with the guidelines of BSL-2, disinfect all materials that enter in contact with the lentivirus by soaking in 10% chlorine bleach for 30 min before disposing.
ii. For lentivirus production of another gRNA (gRNA1-gRNA5), repeat steps B1c-B1k.

2. Transduction of F98 cells *in vitro*
   a. The day before transfection, seed approximately 100k F98 cells into a well of 6-well plate with 2 ml complete growth medium, and distribute the cells evenly across the well.
   b. Thaw the lentivirus solution, add the appropriate volume of polybrene (1:1,000 ratio) and slowly pipet to mix.
   c. Replace the culture medium with 1 ml of lentivirus solution, then place back in the incubator.
   d. Replace the culture medium with fresh one (2 ml), 12 h after transfection.

   Note: To achieve higher transcription of Cas9 and gRNA, it is optional to repeat the steps B2b-B2d (repeat the addition of lentivirus solution to the same cells).
   e. Validate mCherry expression in F98 cells with a fluorescent microscope 72 h after the transfection.
   f. Extract the genomic DNA 72 h after lentivirus transfection with the Universal Genomic DNA Kit, according to the manufacturer's protocols.
3. gRNA evaluation via T7 endonuclease assay
   a. Amplify by PCR 600 to 1,000 bp fragments flanking the cutting site by SaCas9. Briefly spin and mix each component well before use. Set up a reaction as follows:

Q5 Hot Start High-Fidelity 2× Master Mix	2.5 μl
10 μM Forward Primer	1.25 μl (0.5 μM)
10 μM Reverse Primer	1.25 μl (0.5 μM)
Template DNA	500 ng
Nuclease-free H_2O	to 25 μl

Primer sequences of gRNAs:

gRNA-1-F	GATTTTGGATCATTGTT
gRNA-1-R	CCTGTGTCAAAGTCTCA
gRNA-2-F	GCTGGTGCTGTTTCTGG

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gRNA-2-R	CAAATCACCTGCCTTCT
gRNA-3-F	TAAGGTGCCCTGTATGA
gRNA-3-R	GTCGGTAAACTAAGTAAATGTG
gRNA-4-F	TGTGCCGTATTCAGTCC
gRNA-4-R	CTGCGTCCAAACATAAAC
gRNA-5-F	CCAGATGCTGCGTCCAA
gRNA-5-R	CAACAACCCGTTTCTTTC

Note: Primers should be offset from the center of the amplicon to ensure that digestion results in two DNA fragments of different sizes.

b. Briefly spin to collect all liquid to the bottom of tube, then begin PCR with the following program:

Initial Denaturation	98°C	30 s
Denaturation	98°C	5 s (35 cycles)
Annealing	62°C	10 s (35 cycles)
Extension	72°C	20 s (35 cycles)
Final Extension	72°C	2 min

Optional: run a portion of the PCR product on agarose gel to confirm correct amplification.

c. To generate heteroduplex between PCR products, set up a reaction as follows:

PCR Reaction	5 µl (<250 ng)
10× NEBuffer 2	2 µl
Nuclease-free H_2O	12 µl

Note: Cleavage may be incomplete if too much PCR product is added to the heteroduplex digestion reaction.

d. To denature and anneal the products, set up a program as follows:

Denaturation	95°C	5 min
Annealing 1	95-85°C	-2°C/s
Annealing 2	85-25°C	-0.1°C/s
Hold	4°C	

Note: Alternatively, the sample can be heated to 95°C for 10 min and cooled down to room temperature slowly.

e. To digest heteroduplexes, set up a reaction as follows:

Annealed PCR Product	19 µl
EnGen T7 Endonuclease I	1 µl

f. Mix well and briefly spin, incubate the reaction at 37°C for 15 min.

g. Add 1 µl of Proteinase K, and incubate at 37°C for 5 min to stop heteroduplex digestion.

h. Run the products on a 2% agarose gel to analyze DNA fragments, and choose the gRNA with the highest efficiency for gene editing on target locus (Figure 2).

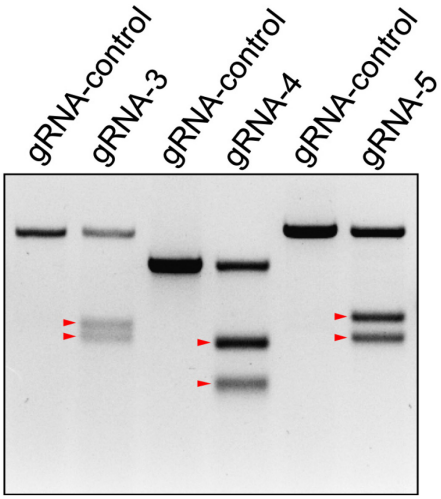

Figure 2. T7 endonuclease assay for genome modification.

SaCas9-mediated DNA cleavage in F98 cells transfected with indicated gRNA. Fraction cleaved is indicated by red triangles. Use following formula to calculate the estimated % modification: % modification = 100 × [1-(1-fraction cleaved)$^{1/2}$].

C. Stereotaxic AAV injection

1. Anesthetize the rat (230 to 250 g) with 1% pentobarbital sodium (intraperitoneally, 0.1 g/kg).
   Note: Anesthesia is confirmed by absence of response to a tail pinch.
2. Shave the scalp from eyes to ears, then position the rat in a stereotaxic instrument.
   Note: It is vital to align the skull along the anteroposterior and mediolateral axis via zygomatic ear bars.
3. Sterilize the surgical field sequentially with cotton swabs dipped in iodine and alcohol.
4. Make an incision along the midline with a scalpel, and scrape away tissues and blood below the incision with a cotton swab.
5. Use a microsyringe fixed on the right arm of the stereotaxic instrument to mark the location of the bregma and requisite injection site: IL [anteroposterior (AP): 2.8 mm; mediolateral (ML): ±0.5 mm; dorsoventral (DV): −4.5 mm], amygdala (AP: −2.8 mm; ML: ±4.8 mm; DV: −8.4 mm).
6. Drill small holes above the requisite coordinates, press the hole with cotton swab to stop potential bleeding.
7. Inject 1 μl of rAAV2-retro-hSyn-Cre-P2A-GFP to the amygdala, and 1 μl of the mixture of AAV9-TRE3G-DIO-SaCas9-3×flag and AAV9-c-fos-rtTA-U6-gRNA (1:1 ratio) to the IL, at a flow rate of 0.1 μl/min with a microinjection pump.
8. To enable virus diffusion and prevent potential leakage from the needle track, leave the needle tip in place for 5 min and gradually withdraw after each injection.
   Note: The needle tip can easily become blocked by blood after injection, so it is recommended to clean it with alcohol and saline each time after use.
9. Suture the incision, then scatter wound powder above the suture.
10. Transfer the rats to their home cages and let them recover for one week.

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D. Contextual fear conditioning

1. Three weeks after AAV injection, handle the rats for 30 min for three consecutive days.
2. Place the rat in the conditioning chamber (25 × 25 × 25 cm) for 300 s, deliver shocks to the feet (0.50 mA, 2 s) at 120 s, 180 s, and 240 s, then transfer the rat to its home cage.
3. Two weeks later, feed the rat with doxycycline diet (100 mg/kg) for one day to allow a time window for activity-dependent cell labeling.
4. One day later, place the rat in the conditioning chamber for 300 s to label IL extinction-ensemble cells with SaCas9. Transfer the rat to its home cage, and withdraw the doxycycline diet immediately.
5. One week later, place the rat in the conditioning chamber for 300 s to monitor its fear extinction for three consecutive days.
   Note: Clean the chamber with ethanol prior to introducing the next rat. Freezing is defined as being motionless and indicated as the percentage of total session time.
6. Perform standard histological immunofluorescence (Koffman and Du, 2020) on the brain tissue to verify the expression of SaCas9 after contextual fear conditioning (Figure 3).

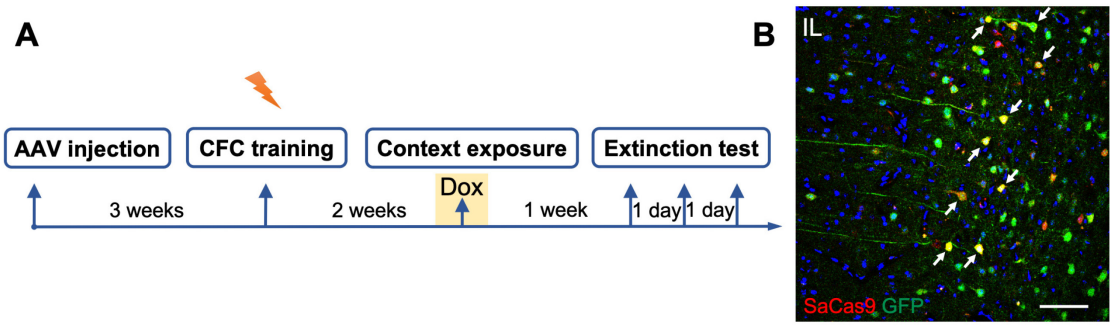

Figure 3. Expression of SaCas9 in IL after contextual fear conditioning.

A. Scheme of behavioral testing. B. Immunofluorescence showing a portion of amygdala-projecting IL neurons (GFP^+) also stained with activity- and Cre-dependent SaCas9. Scale bar, 100 μm.

Data analysis

Perform immunofluorescence to verify virus infection after each experiment, and exclude animals with inappropriate virus infection from the analysis. Data are expressed as means ± SEM and analyzed using GraphPad Prism. Perform statistical analysis of two experimental groups using two-tailed Student's *t*-tests and comparisons between two groups with different time points using two-way analysis of variance (ANOVA), with $P < 0.05$ as statistically significant.

Recipes

1. Complete growth medium for 293T and F98 cells
   a. FBS, heat inactivated at 50°C for 30 min before making medium.
   b. Prepare medium by adding 1% volume of penicillin-streptomycin and 10% volume of FBS to DMEM.
   c. Filter sterilize, store at 4°C for up to 1 month.

Acknowledgments

This protocol was used to obtain a portion of data published in *Science Advances* (Sun *et al*., 2020; DOI: 10.1126/sciadv.aay6687). This work was supported by grants from the National Natural Science Foundation of China (81974166, 31872774, 31371119, 91732107, 81571067, and 81821092), the National Basic Research (973) Program of the Ministry of Science and Technology of China (2014CB548200 and 2015CB554503), the “111” Project of Ministry of Education of China (B07001), the Beijing Natural Science Foundation (5182013), and the Interdisciplinary Medicine Seed Fund of Peking University (BMU2018MX011).

Competing interests

The authors declare that they have no competing interests.

Ethics

All experimental procedures complied with the guidelines of the Animal Care and Use Committee of the Peking University Health Science Center.

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