# Retrovirus-mediated single-cell gene knockout technique in adult newborn neurons *in vivo*

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Single-cell genetic manipulation in an intact brain environment is an informative approach to study molecular mechanism of adult neurogenesis. Here, we describe a protocol for retrovirus-mediated single-cell gene knockout in adult new neurons *in vivo*. A gene of interest is disrupted in adult floxed mice by a vector based on the Moloney murine leukemia retrovirus, expressing Cre recombinase. High-titer retrovirus is prepared by transfecting plasmids into the HEK293T cells and by concentrating the supernatant containing virus. The retrovirus is stereotaxically injected into the dentate gyrus. Cre recombinase is transduced and expressed in a small fraction of adult new neurons in an intact environment, and the gene knockout is highly efficient within the transduced neurons. Virus preparation takes 7 days, but virus injections take less than 1 h per mouse. By changing the survival time of the mice after the injection, one can analyze the effects on new neurons at different ages.

#### INTRODUCTION

In the adult mammalian brain, new neurons are generated and incorporated into the existing neural circuits in restricted regions including the dentate gyrus and the olfactory bulb<sup>1</sup>. Although studies have revealed a large body of information on its molecular mechanisms, an overall picture of how adult neurogenesis is regulated is still largely unclear<sup>2</sup>. Most of these studies have utilized global manipulation such as drug/virus infusion and conventional genetically modified mice. Although these approaches can indicate that a gene or protein of interest is somehow related to adult neurogenesis, interpretation of results is inevitably confounded by possible global alteration of the surrounding environment, for example, observed effects could be side effects caused by disturbance in the activity pattern of neighboring neural circuits or signaling from other neurons.

Therefore, it is informative to examine the behavior of single neurons when a small number of neurons are manipulated leaving their surrounding environment intact. To achieve this, we developed a retrovirus-mediated single-cell knockout technique in adult new neurons in vivo<sup>3</sup>. The principle of the technique is to deliver Cre recombinase<sup>4</sup> selectively to adult new neurons<sup>5,6</sup>, using a retroviral vector, in mice floxed for a gene of interest. Floxed genes are inserted with target sequences of Cre recombinase, called the loxP sequences. Cre recombinase recognizes the loxP sequences and removes the gene of interest. Using this technique, we prevented the expression of NMDA-type glutamate receptor (NMDAR), which receives signals from synaptic inputs, only in a small number of new neurons<sup>3</sup>. The survival of new neurons was affected in the absence of NMDAR, whereas the survival of wild-type new neurons in the same animals was not. Thus, we demonstrated that NMDAR-mediated regulation of new neuron survival is input-dependent and cell-specific. Further, as shown in this same study, the single-cell knockout technique is advantageous not only to reveal the cell-specific regulation, but also to indicate a competitive mechanism in the survival regulation, which is difficult to address with conventional global manipulations.

The single-cell gene knockout technique was designed specifically to examine the behavior of manipulated single cells in intact environment. Therefore, this technique is suited for single-cell analyses such as morphological and patch-clamp analysis. On the other hand, because only a small number of new neurons are manipulated and the majority of new neurons are intact, this technique is not adequate for whole-animal studies such as behavioral experiments.

One limitation of this single-cell knockout technique is the requirement of floxed mice generated for a specific gene of interest. However, currently, many such animals exist and laboratories continue to produce conditional knockout mice using the Cre/ loxP system<sup>4</sup>. It is advantageous in that this single-cell knockout technique is able to use these floxed mice produced for different purposes with a small additional effort. An alternative method, which is possibly more generally applicable, is retrovirus-mediated RNA interference. This approach was used to knock down a Na+-K<sup>+</sup>-2Cl<sup>-</sup> transporter, NKCC1, in adult new neurons<sup>7</sup>. One disadvantage of this method is that the expression of a gene of interest cannot be suppressed completely, in contrast with the complete elimination of the gene of interest in the knockout approaches. The other problem of this RNA interference-based approach is the difficulty in verifying that an observed effect is really owing to knockdown of a gene of interest. For this purpose, it is necessary to use multiple sequences targeting the same gene and/or to perform rescue experiments with a homologous gene that is not affected by the sequences. This requires constructing multiple viral vectors and performing experiments with them. On the other hand, for the single-cell knockout technique, this verification can be more easily achieved by injecting the same viral vector in wild-type mice as we performed in the previous study<sup>3</sup>. A possible compliment to the retroviruses expressing Cre in floxed animals is to use promoters specific for new neurons with viral vectors such as lentiviral or adenoviral vectors. In this case, the specificity of promoters must be extremely high. Because new neurons are a minority in the adult brain (estimated  $\sim 3\%$  in the dentate gyrus) and intermixed with a

large number of pre-existing mature neurons, even a small degree of nonspecificity could affect a much larger number of mature neurons than new neurons (T. Sawai, A. Tashiro & F.H.G., unpublished observation).

All of these methods require high titer virus preparation and precise delivery of virus into the dentate gyrus. To prepare concentrated retrovirus for single-cell knockout, one needs an efficient transfection protocol and plasmid DNAs that are required for virus assembly, including the recombinant viral vector that contains the transgene, a plasmid that expresses gag and pol, and a plasmid that encodes an envelope protein.

The wild-type retrovirus genomes contain three genes, gag, pol and env, encoding the structural, enzymatic and envelope proteins required for virus replication, respectively. In the recombinant retroviral vector system, these genes were separated from the virus genome so that the amplified virus is replication incompetent<sup>8</sup>. This has served two purposes. First, it is safer. Second, the gene expression is restricted to virus-transduced cells at the time of infection and the progeny of these cells, which allows the tracing of transduced cells. VSV-G is the envelope glycoprotein of the rhabdovirus vesicular stomatitis virus, and has been used to replace the envelope proteins of other viruses to increase the host range. In addition, VSV-G-pseudotyped virus is more stable under highspeed centrifugation<sup>9</sup>.

Typically, a recombinant retroviral vector contains viral long terminal repeats at ends, the viral packaging signal, the promoter and gene of interest, and the Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) (**Fig. 1**). Our vector is based on the pCL system by Naviaux *et al.*<sup>10</sup>. It uses the cytome-galovirus (CMV) promoter to drive the transcription of the modified viral genome, which is based on the Moloney murine leukemia virus (MoMLV). The compound CAG promoter that contains chicken actin promoter, CMV enhancer and a large



**Figure 1** | A schematic structure of the recombinant retroviral genome of CAG-GFP/cre. This vector is based on the MoMLV. The transcription of the viral genome is under the control of CMV promoter to allow high-titer virus preparation with HEK293T cells. The expression of GFP/cre is driven by the compound promoter CAG, which contains the minimum enhancer sequence of CMV, chicken actin promoter and a synthetic intron. LTR: long terminal repeat;  $\Psi$ : viral packaging signal; WPRE: Woodchuck hepatitis virus post-transcriptional regulatory element.

synthetic intron was used to allow the ubiquitous expression of GFP-fused Cre recombinase (GFP/Cre)<sup>11</sup> in transduced cells<sup>3,6</sup>. The WPRE element is not an essential element of the virus backbone, but it has been reported to stabilize the transcripts and increase the expression level of transgene by several folds<sup>12</sup>.

Because all three plasmid DNA need to be present in the same cell for the assembly of the virus, the transfection efficiency is critical for generating high-titer virus preparation. We typically use the HEK293T cells for virus preparation and have used two methods in our laboratory, lipofectamine 2000 and calcium phosphate precipitation. The protocol with lipofectamine 2000 (Invitrogen) is highly consistent, less labor-intensive, but more expensive. In comparison, our protocol for calcium phosphate precipitation has been less consistent. We would recommend a test transfection to determine the efficiency of the reagents before starting a retrovirus preparation with the calcium phosphate precipitation protocol.

This protocol can be used for other applications of retrovirus to manipulate new neurons in the adult brain. For example, one can overexpress mutated genes, such as constitutively active or dominant-negative mutants or conditionally express genes by using regulatable promoters, such as the tetracycline-transactivator system<sup>13</sup>. A flow diagram of the procedure is shown in **Figure 2**.

# MATERIALS

REAGENTS

- •6–8-week-old floxed mice **!** CAUTION All animal experiments are to be performed in accordance with relevant authorities' guidelines and regulations.
- Plasmid DNA: pCMV-gp (a gift from the laboratory of Inder Verma at Salk Institute), pCMV-vsv-g and CAG-GFP/cre<sup>3</sup>. The plasmids are available from the corresponding author
- Culture medium: Dulbecco's modified Eagle's medium (high glucose, with glutamine) or Iscove's modified Dulbecco's medium (Gibco, no. 12440-053) supplemented with 10% (vol/vol) fetal bovine serum
- Transfection reagent: lipofectamine 2000 (Invitrogen, 11668-019), Opti-MEM (Invitrogen, 31950-062 or 31985-070)
- Alternative transfection reagent: 2× HEBS, 280 mM NaCl, 10 mM KCl, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 12 mM D-glucose, 50 mM HEPES (acid-free), pH 7.05, adjust with NaOH or HCl, filter sterilize (do not autoclave), 2 M CaCl<sub>2</sub>, filter sterilize (wet filter with a few drops of water to allow filtration)
  Dulbecco's PBS (Invitrogen, 14287-072)
- Anesthetics (10 mg ml<sup>-1</sup> ketamine and 1 mg ml<sup>-1</sup> xylazine in 0.9% NaCl)
   CAUTION Controlled substances (e.g., ketamine) must be locked up when not in use and a controlled substance usage log must be kept on them.

- •70% ethanol
- Cotton tips
- Puralube Vet Ointment (Pharmaderm, NDC 0462-0211-38)
- Tissue adhesive (3M Vetbond, 1469SB)
- EQUIPMENT
- Biosafety level-2 facility **! CAUTION** For general regulations of Biosafety level-2 facility, please see pages 21–27 of Biosafety in Microbiological and Biomedical Laboratories (http://www.cdc.gov/od/ohs/pdffiles/4th%20BMBL.pdf).
- •14 ml polypropylene tubes (Falcon 352059)
- Ultracentrifuge (Beckman Coulter)
- Rotors for ultracentrifuge: SW32 Ti or SW28, SW55 Ti (Beckman Coulter)
- Polyallomer tubes 25  $\times$  89 mm (Beckman Coulter, cat. no. 326823) or
- $25 \times 89$  mm konical (cat. no. 358126)
- Polyallomer tubes  $13 \times 51 \text{ mm}$  (Beckman Coulter, cat. no. 326819)
- Small animal stereotaxic frame (Kopf)
- Microsyringes (Hamilton, cat. no. 87925)
- Needles for microsyringes, 33 gauge (Hamilton, cat. no. 7762-06)
- Electric drill (Dremel, model 395 T6)
- Drill bur, size no. 1 (Henry Shein, cat. no. 100-7176)
- · Electric hair trimmer or small scissors

#### PROCEDURE

#### Preparation of high-titer retrovirus

1 Viruses can be prepared by lipofection (option A) or calcium phosphate transfection (option B).

**!** CAUTION Pseudotyped MoMLV-based retrovirus is capable of infecting human cells through contact. Gloves and protective clothing are required for working with retroviruses. Extra caution should be taken to avoid spill and splash when handling retrovirus-containing material. Retroviruses are labile and easily decontaminated by ethanol, detergent or bleach. Working area should be decontaminated with ethanol or bleach after any spill or after the completion of work.

**!** CAUTION Replication incompetent retroviruses are considered to be infectious material, and biosafety level-2 laboratories are used for virus preparation and manipulation. Follow the guidelines of NIH/CDC (reference: http://www.cdc. gov/od/ohs/pdffiles/4th%20BMBL.pdf) and your institution for biosafety level-2 laboratories. Stream-sterilize all solid waste before disposal. Although cells and media are not infectious until the transfection step, we perform the entire procedures of virus preparation in the biosafety level-2 laboratories to avoid problems associated with transferring materials, such as contamination.

(A) Retrovirus preparation with lipofectamine 2000

(i) Day 1: The day before transfection, plate  $5 \times 10^6$  cells per 100 mm plate for a total of 12 plates. This roughly equals 1/3 or 1/4 of one 100 mm plate of confluent





293T cells. Use medium including antibiotics when maintaining the 293T cells, but use medium without antibiotics at this step. Use 10 ml medium for each plate.

- (ii) Transfection (day 2) TIMING ~ 6 h: Plan to transfect cells in the morning because there is a 5-h waiting time to change media. Prepare eight tubes (14 ml polypropylene tubes); add 2.4 ml Opti-MEM to each tube.
- (iii) To four tubes, add 45 µg DNA mix (containing 22.5 µg CAG-GFP/cre, 15 µg pCMV-gp and 7.5 µg pCMV-vsv-g) and mix well by tabbing the tube.
- (iv) To the other four tubes, add 150 µl lipofectamine 2000. Mix by tabbing the tube.
   ▲ CRITICAL STEP Incubate at room temperature (20–25 °C) for no longer than 5 min.
- (v) Mix one tube of DNA (Step 1A(iii)) with one tube of lipofectamine 2000 (Step 1A(iv)).
- CRITICAL STEP Transfer DNA into the lipofectamine tube, pipette up and down for 2–3 times, and mix well by tabbing the tube. Do this one by one, and you will have four tubes of DNA–lipofectamine mix with approximately 4.8 ml in each tube. Each tube transfects three of 100 mm plates.
- (vi) Incubate at room temperature for 25-30 min.
- (vii) Transfer 1.6 ml of the DNA/lipid solution from Step 1A(vi) onto each plate of 293T cells containing normal culture medium without antibiotics, dispense the mix dropwise and have them evenly distributed on the surface of the cells. At the end, gently rock the plate and place the plate back to the incubator. You can do this in set of three.
- (viii) Incubate for 5 h at 37 °C, 5% CO<sub>2</sub>, then replace supernatant with 10 ml fresh media. Media containing antibiotics can be used at this step.
- (ix) Concentrate virus (day 4): collect virus. Start early. A total of  $\sim 6$  h is needed to complete the whole procedure, but there are some waiting times.

**!** CAUTION Handle retrovirus material with caution and avoid spills. Use bleach to decontaminate hazardous liquids (10% final concentration for 30 min). All plasticware are to be stream-sterilized before disposal.

- (x) Collect the supernatant in 50 ml conical tubes. Centrifuge at 1,000g for 2-3 min to remove cell debris.
- (xi) Filter the supernatant through 0.22  $\mu m$  filter top. It is not necessary to pre-wet the filters.
- (xii) Transfer filtered supernatant into four ultracentrifuge polyallomer tubes (25  $\times$  89 mm, tubes A). Add PBS to the tubes so that the top of the solution is  $\sim$  0.5 cm from the top of the tube.
- (xiii) Centrifuge at 65,000g and 4  $^{\circ}$ C using the rotor SW32 Ti or SW28 for 2 h.
- (xiv) Remove the supernatant with Pasteur pipette.

**!** CAUTION Connect the flask to the venting system through a filter (Vacushield Vent Device, Pall Corp. no. 4402).

- (xv) Resuspend virus with 0.7 ml PBS for each tube ( $\sim$  2.8 ml total). Pipette up and down for 20–30 times—adjust pipetteman to 0.5 ml to minimize air bubbles from pipetting. Transfer all to one 4 ml ultracentrifuge tube (13  $\times$  51 mm, tube B). Sequentially wash tubes A with 0.7 ml PBS and transfer to tube B (3.5 ml now in tube B).
- (xvi) (Optional) Add 0.5 ml 20% sucrose cushion (made in PBS and filter-sterilized) to tube B.

- (xvii) Fill the tube with more PBS so that the top of solution is  $\sim$  0.5 cm from the top of the tube. Spin at 65,000*g* using the rotor SW55 Ti, 4 °C for 2 h.
- (xviii) Resuspend the final pellet in 80  $\mu$ l PBS by vortexing for 30 s and then by pipetting. This procedure is enough to resuspend the virus, although white or brown sticky pellet sometimes remains after resuspension. Transfer the virus to a 0.5 ml tube (tube C); wash tube B once with 20  $\mu$ l PBS and transfer the solution to tube C. Spin tube C briefly and transfer the supernatant to a new tube. Aliquot in 5–10  $\mu$ l and store at -80 °C.

**!** CAUTION It is important that the virus solution is stored in small aliquots and stored at -80 °C. Virus solution can be at -80 °C for at least 1 year without significant change in virus titer. Do not re-freeze virus solution after thawing. The virus solution can be stored at 4 °C if an experiment is planned within a week.

## (B) Retrovirus preparation by calcium phosphate precipitation

(i) Again, the transfection efficiency with calcium phosphate precipitation has not been consistent in our hands. It is better to conduct a test transfection before starting the preparation. Ideally, a transfection efficiency of >50% is needed to have a good titer. On day 1, plate 293T cells at  $8-10 \times 10^6$  per 150 mm plate. Prepare 10–20 plates for virus preparation. Use 15 ml media for each plate. Incubate cells with 10% CO<sub>2</sub>.

## Calcium phosphate tranfection (day 2, late afternoon, 4-6 pm)

- (ii) For each of the five plates, add to a 50 ml conical tube (3.2-X) ml sterile ddH<sub>2</sub>O, where X is the volume of your DNA mix including 150 μg CAG-GFP/cre, 100 μg pCMV-gp and 40–50 μg pCMV-vsv-g.
- (iii) Add DNA to the water and mix by tabbing the tube.
- (iv) Add 1.8 ml CaCl<sub>2</sub> solution to the tube, mix by pipetting up and down three times. Tab on the tube for a few times. The total volume of  $DNA/CaCl_2$  mix is 5 ml.
- (v) Add 5 ml 2× HEBS solution to the DNA/CaCl<sub>2</sub>.
   ▲ CRITICAL STEP Mix—add from high above (for example, position the tip of the pipette a little above the opening of the conical tube).
- (vi) Mix by pipetting up and down three times and then by bubbling air for 30 s.
- (vii) Incubate the mixture for 10 min at room temperature.
- (viii) Add 2 ml of the mix to each plate.

**CRITICAL STEP** Add dropwise and evenly on the plate.

- (ix) Distribute evenly by gently rocking the plate.
- (x) Incubate at 37  $^\circ$ C in 5% CO<sub>2</sub> overnight.
- (xi) Day 3 early morning (8–10 a.m.): change media, and move cells back to 10% CO<sub>2</sub> incubator.
- (xii) Day 4: collect the supernatant with 50 ml conical tubes. Add fresh media to plates and put cells back to 10% CO<sub>2</sub>.
   **CAUTION** Use bleach to decontaminate hazardous liquids. All plasticware are to be stream-sterilized before disposal.
- (xiii) Day 5: collect the supernatant again and discard cells.
- (xiv) Concentrate virus following Steps 1A(ix)–(xvii). Two ultracentrifuges might be needed because of the large volume of total supernatant. For example, a total of 300 ml supernatant will be collected if ten of 150 mm plates are used for virus preparation. Each rotor can only accommodate 180–200 ml.

## Determine the titer of the virus

**2**| Seed 10<sup>5</sup> 293T cells per well in 24-well plate 1 day ahead. This is about 1/200 of the amount of cells from one confluent 10 cm dish. Seed four wells for one virus preparation.

**!** CAUTION Vsvg-pseudotyped MoMLV-based retrovirus is capable of infecting human cells through contact. Gloves and protective clothing are required for working with retroviruses. Extra caution should be taken to avoid spill and splash when handling retrovirus-containing material. Retroviruses are labile and easily decontaminated by ethanol, detergent or bleach. Working area should be decontaminated with ethanol or bleach after any spill or after the completion of work.

**3** Steps 3 and 4 are carried out in the virus laboratory. Perform a serial dilution  $(10^2, 10^3, 10^4 \text{ and } 10^5)$  of the virus and transfer 10 µl of each dilution to one well so that the final dilution series are  $10^1$ ,  $10^2$ ,  $10^3$  and  $10^4$ .

**4** Count the number (*n*) of fluorescent clusters under the fluorescent microscope 2–3 days after viral infection. The virus titer is ( $n \times dilution/10$ ) colony-forming unit (c.f.u.) per µl. Typically, one could obtain a titer of  $10^4-10^5$  c.f.u.  $\mu l^{-1}$ , that is  $10^7-10^8$  c.f.u.  $m l^{-1}$ . p24 measurement by real-time PCR or ELISA can be an additional or alternative option to measure the titer of the retrovirus<sup>14</sup>.

## Injection of virus vectors into the dentate gyrus

**5** Inject anesthetics into 6–8-week-old floxed mice of interest. Wait for 5 min so that the mice do not respond to pinching with tweezers.

**!** CAUTION All animal experiments are to be performed in accordance with relevant authorities' guidelines and regulations.



**Figure 3** | Stereotaxic injection. (a) After a mouse is mounted on a stereotaxic frame and its skull is exposed, the bregma is localized (arrowhead). Antero-posterior (AP) axis is indicated on left. (b) The needle tip is moved onto the bregma (arrowhead). (c) The tip is moved posteriorly and laterally to an appropriate stereotaxic coordinate. The position of the tip (arrow) is marked by a marker pen. (d) A small hole (arrow) is made in the skull by using an electric drill. (e) The needle is brought down to an appropriate coordinate through the hole (arrow).

**!** CAUTION Retrovirus is infectious to human by contact. Wear Latex gloves when handling the virus. Avoid spills. Thoroughly decontaminate equipment with 70% alcohol after use.

6 Shave a small area on the head with a trimmer. Apply eye ointment (Pura lube Vet Ointment) to prevent eyes from over-drying.

7 Cut the skin over the skull about 1 cm. Wipe blood from the wound.

8 Mount the mouse onto a stereotaxic frame.

**CRITICAL STEP** Orienting the head straight in terms of the antero-posterior axis and horizontally is important for consistent injection into the target area.

9 Move the tip of injection needle to the bregma. See Figure 3.

**10** Move the needle tip to 1.5 mm lateral and 2 mm posterior. Mark the position with a marker pen. The stereotaxic coordinate shown here (Steps 10 and 13) is for 6–8-week-old floxed NR1 mice and C57Bl/6 mice used by Tashiro *et al.*<sup>3</sup>. The coordinates need to be adjusted according to the line and age of mice used for each study.

- **11** Move the needle out and make a small hole on the skull using an electric drill.
- **12** Dip the needle tip into virus solution and load 1.5  $\mu$ l of solution into the syringe.

13 Move the needle tip to the hole and set it to the level of skull surface.CRITICAL STEP Move the needle tip down by 2.3 mm.

- **14**| Inject a total of 1.5 μl of virus solution.
- **A CRITICAL STEP** We manually inject 0.05  $\mu$ l every 6 s.

**15** After finishing the injection, wait for 1 min to prevent the injected solution from flowing back through the needle track.

**16** Move the needle tip 1 mm up and then wait for another 1 min.

17 Move the needle tip 1 mm up and the tip is out of the brain. Rinse the needle with 70% ethanol and PBS sequentially.

**18** Close the skin using tissue glue or by sewing with thread. Place the mouse in a cage on top of a warm blanket until it is fully alert. Transfer the mouse back to home cage.

**19** Analysis. A variety of single-cell analysis is possible. For example, neuronal differentiation and morphogenesis can be

Figure 4 | New neurons expressing GFP/cre in the adult dentate gyrus.
(a) A new neuron in adult ROSA26 GFP reporter mice. Because GFP/cre is localized in the nucleus, GFP fluorescence (green) in the cytoplasm, most evidently in dendrites, indicates the occurrence of Cre-mediated recombination in the new neuron. Red: anti-NeuN immunostaining.
(b) A new neuron in adult floxed NR1 mice, cotransduced by two viral vectors (CAG-GFP/cre and Red fluorescent protein (RFP)).
A subpopulation of new neurons coexpressed GFP/cre (green) and RFP (red).
Blue: anti-Prox1 immunostaining. Scale bar, 30 µm in (a), 60 µm in (b).



analyzed in fixed brain sections with immunostaining<sup>3,5,6</sup>. Electrophysiology and morphological dynamics can be studied in live brain slices<sup>3,5</sup>. Importantly, depending on survival time after virus injection, new neurons at different maturational stages can be analyzed<sup>3,6</sup>. We have analyzed from 1 day to 14 months after virus injections.

## • TIMING

Steps 1A(i)—(xviii): virus preparation, 4 days (30 min for day 1, 6 h for day 2 and 6 h for day 4) Steps 1B(i)–(xiv): virus preparation, 5 days (30–60 min for day 1, 60 min for day 2, 30 min for day 3, 30 min for day 4 and 6–8 h for day 5) Steps 2–4: 4 days (15 min for day 1, 30 min for day 2 and 10–20 min for day 4) Steps 5–18: virus injection, 15 min to 1 h for each mouse (depending on the experimenter's experience)

## ? TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

**TABLE 1** | Troubleshooting table.

Problem	Possible reason	Solution
Step 1A(xiii)—tubes deform after spinning	Adapters are not used with conical polyallomer tubes	Use adapters
	Tubes are not filled with solution	Fill the tubes with PBS so that the surface of the solution is $\sim 0.5~{\rm cm}$ away from the tube opening
Step 1B—low transfection efficiency	pH of 2 $\times$ HBS might not be optimal	Make several $2 \times$ HBS solutions and test the efficiency of each of them. Use the one that gives the maximum transfection efficiency (at least 50%)
No GFP/cre-positive cells found	The titer of the virus is too low	Prepare a new batch of virus with a high titer
GFP/cre-positive cells only outside of targeted area	Injection into wrong area	Correctly mount mice on the stereotaxic frame Find a good stereotaxic coordinate by injecting a dye (e.g., gel-loading dye for electrophoresis) into the same line of mice at the same age as used for specific studies. The size of brain is different between lines of mice and different ages

## ANTICIPATED RESULTS

CAG-GFP/cre was injected into ROSA26 GFP reporter mice and floxed NR1 mice (**Fig. 4**). In ROSA26 GFP reporter mice, we found that Cre-mediated recombination occurred in more than 97% of GFP/cre-expressing neurons. In fNR1 mice, none of the neurons examined responded to an NMDA receptor agonist. Thus, in these mice, recombination efficiency was quite high. The density of GFP/cre-expressing new neurons is higher at a position closer to injection sites, and the average density typically ranged from 4 to 10 cells per 40- $\mu$ m-thick coronal sections with an injection of 1.5  $\mu$ l virus (10<sup>7</sup>–10<sup>8</sup> c.f.u. ml<sup>-1</sup>). As controls, one can use CAG-GFP in separate mice or CAG-RFP in the same mice. With the latter, an issue of variability from animal to animal can be reduced.

**COMPETING INTERESTS STATEMENT** The authors declare that they have no competing financial interests.

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