Chapter 13

Gene Knockout in the Developing Brain of Wild-Type Rodents by CRISPR In Utero Electroporation

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Abstract

CRISPR/Cas9 constructs can be delivered by in utero electroporation to knock out a gene of interest in neurons of the developing brain in wild-type rodents. This approach allows for high-throughput genetic screening, circuit-specific gene knockout, and knockout cell phenotyping using sparse labeling within a wild-type in vivo context. Here we outline the methods and steps of designing guide RNAs in silico, cloning guide RNAs into plasmid backbones, and introducing these plasmids into the developing mouse cortex and hippocampus.

Key words CRISPR, Genome editing, In utero electroporation, Knockout, Brain development

1 Introduction

Neurodevelopmental disorders are often linked to mutations in genes that are expressed early in brain development. An alternative to using knockout animals produced through transgenic technologies is to genetically target cell types of interest directly in the developing brain of non-transgenic animals. This approach can be advantageous when time and throughput requirements do not allow the generation of mouse strains. Additionally, this approach can be a valuable alternative when germline strains and conditional strains are not viable or develop undesired systemic conditions. Targeted gene knockout in cells of wild-type animals has become experimentally feasible with the introduction of RNA-guided CRISPR/Cas nucleases as genome editing agents [1, 2]. In the developing brain, we and others have used CRISPR/Cas approaches for acutely knocking out a gene [3–5], knocking in a fusion protein or epitope tag [6], and editing single-nucleotide patient variants [7], all with cell-type specificity circumventing germline transmission breeding strategies.

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Brain development occurs in phases over distinct time points, where progenitor cells give rise to specific neuron types. Progenitor cells typically line the ventricular system around which the central nervous system develops. We take advantage of the characteristic locations and developmental timing of distinct progenitor pools to target region-specific neuron subtypes using in utero electroporation [8–11]. In utero electroporation can deliver plasmid DNA to a subpopulation of progenitor cells by allowing it to enter the cells by the application of a transient electric field which enables negatively charged DNA injected into the ventricle to cross the cell membrane of progenitor cells lining the ventricle. Specific subsets of neurons can be targeted by selecting specific gestation times and by orientation of the electrode relative to the brain's surface. For example, cortical layer 2/3 excitatory neurons can be targeted at embryonic day (E) 15 when the electrode is positioned with the positive pole on the dorsal half of the brain, leading DNA injected in a lateral ventricle into progenitor cells of the dorsal pallium that give rise to cortical glutamatergic cells [10, 12, 13]. Conversely, injecting DNA into the lateral ventricle and orienting the electric field so that the positive pole faces the ventral surface, will lead DNA into the progenitor cells of the ventral pallium, which give rise to cortical GABAergic neurons [14, 15]. By targeting subpopulations of progenitor cells, in utero electroporation provides a technique to sparsely label neurons and introduce payloads via plasmids into neurons during early development.

Using in utero electroporation, we can introduce CRISPR/Cas9 constructs into progenitor cells, thereby producing a population of knockout cells in an otherwise wild-type brain. Cas9 is an RNA-guided nuclease from the clustered regularly interspaced short palindromic repeats (CRISPR) adaptive immunity discovered in microbes [16, 17]. Cas9 is guided by a short RNA sequence 20 nucleotides in length that is complementary to the target sequence directly upstream of a protospacer adjacent motif (PAM) sequence specific to the type of CRISPR system being used. In this protocol, we are using the system derived from *Streptococcus pyogenes* (spCas9 or Cas9) which uses a 5′-NGG PAM. Using a designed guide RNA (gRNA), Cas9 can be targeted to a specific gene locus and introduce a double-strand break in the DNA [18], which is subsequently repaired by cellular machinery in ways that lead to insertion or deletion of nucleotides (indels) anywhere from 25% to 95% of the time depending on the specific locus [19]. These indels often result in frame-shift mutations disrupting the Open Reading Frame (ORF) of the gene thereby resulting in protein products that are non-functional and typical of conventional knockout approaches. CRISPR/Cas9 has been used in many other contexts to create full knockout animal lines and cell-type specific knockouts. Combining CRISPR/cas9 and in utero electroporation is a powerful technique allowing for the knockout and labeling of a

sparse population of neurons of the same subtype, arising from the same progenitor pool. These neurons can then be studied in the context of a mostly wild-type environment which can circumvent lethal phenotypes of knockout lines, and without having to create transgenic lines for each gene.

The following describes how to design and clone gRNA plasmids, and introduce these plasmids into the developing rodent cortex via in utero electroporation to knockout a gene.

2 Materials

2.1 Design and Preparation of Plasmids

1. Plasmids:
   - P396 gRNA backbone plasmid (pJ2.U6<gRNA_scaffold, Addgene plasmid #226288).
   - A Cas9 plasmid with a promoter that expresses in the embryonic brain (i.e., CAG or EF1α promoters, e.g., Addgene plasmid #51142).
   - A fluorescent protein (FP) plasmid with a strong promoter that expresses in the embryonic brain (i.e., CAG promoter) to facilitate screening.
2. PCR tubes with caps.
3. Nuclease-free water (e.g., Invitrogen).
4. BbsI restriction enzyme (e.g., NEB BbsI-HF).
5. T4 DNA Ligase (e.g., NEB T4 DNA ligase).
6. Ligase Buffer (e.g., Promega T4 DNA ligase 10× buffer).
7. Chemically competent bacteria (e.g., One Shot TOP10, Thermofisher Scientific).
8. S.O.C media.
9. Autoclaved glass beads.
10. Carbenicillin or Ampicillin agar plates.
11. Autoclaved pipette tips or toothpicks.
12. Lysogeny broth (LB).
13. Ampicillin sodium salt, dissolved in sterile water to 100 mg/mL.
14. 14 mL round bottom tubes with caps.
15. Miniprep kit (e.g., Quick-DNA Miniprep Plus Kit, Zymo Research).
16. 1 L Erlenmeyer flask, autoclaved.
17. Midiprep kit (e.g., ZymoPURE Express Plasmid Midiprep Kit, Zymo Research).
18. 100% ethanol, molecular grade.

19. 3 M Sodium Acetate (pH 5.2), molecular grade (e.g., Thermofisher Scientific).
20. 1.5 mL Eppendorf tubes, autoclaved.
21. DNA Elution buffer (e.g., Zymo Research).
22. Thermocycler.
23. Heat block set to 42 °C.
24. Heat block set to 37 °C, shaking at 650 rpm.
25. Plate incubator set to 37 °C.
26. Shaking culture incubator set to 37 °C, shaking at 230 rpm.
27. Large centrifuge for midi prep.
28. Vacuum manifold.
29. Refrigerated microcentrifuge.
30. Spectrophotometer (e.g., NanoDrop One, Thermo Scientific).

2.2 In Utero Electroporation

1. E15 or E16 Timed-pregnant mice (e.g., CD1 from Charles River Laboratories).
2. Isoflurane.
3. Oxygen tank and regulator.
4. Isoflurane vaporizer system and induction chamber.
5. 0.1% filtered Fast green FCF in nuclease-free water.
6. Analgesic (e.g., Buprenorphine).
7. Sterile cotton tip applicators.
8. Sterile gauze (or equivalently press' N seal plastic wrap).
9. Hair removal cream (e.g., Veet).
10. Sterile disposable transfer pipettes.
11. Betadine surgical scrub.
12. Sterile surgical gloves.
13. Sterile bench pads.
14. 1/2 cc syringe with 27 G needle.
15. Parafilm.
16. 50 mL conical tubes.
17. Nylon monofilament reverse cutting 3/8 c 12 mm sutures.
18. Sterile 1× Phosphate Buffered Saline (PBS).
19. Alcohol antiseptic pads.
20. Glass capillary tubes, non-heparinized, diameter 1.1–1.2 mm (e.g., Fisherbrand).
21. Eye lubricant (e.g., Puralube Vet Ointment).
22. Electroporator (e.g., Electro Square Porator ECM 830).

23. Tweezertrodes (e.g., BTX company).
24. Water heat pad set to 37 °C (e.g., HTP-1500 heat therapy pump).
25. Dry block heater with 50 mL tube block, set to 40 °C (e.g., VWR, mini block heater).
26. Isoflurane vaporizerPipette puller (e.g., Narishige).
27. Pipette beveler (e.g., Narishige).
28. Dissecting microscope.
29. Sterile surgical scissors, medium and micro.
30. Sterile forceps, blunt and sharp.
31. Sterile suturing tools.
32. Aspirator tube assemblies for calibrated microcapillary (e.g., Sigma-Aldrich).
33. Red heat lamp.
34. Fluorescent stereoscope (e.g., Leica).

3 Methods

3.1 Design and Preparation of CRISPR Knockout Plasmids

In the following steps, we outline how to design and clone gRNAs that are needed to perform gene knockout using spCas9. Several online platforms, including benchling.com, indelphi.giffordlab.mit.edu [20], and CRISPOR.tefor.net [21], allow one to import the genomic sequence of a gene and design gRNAs for gene knockout. Predictions of precision and efficiency are provided to compare different gRNA options. Once gRNA target sequences have been selected, gRNA sequences can be synthesized or ordered using a nucleic acid synthesis provider, as such as those used commonly to synthesize oligos for PCR, and subcloned into a plasmid using common cloning protocols. Here we describe the process of producing gRNA constructs using Golden Gate Assembly (GGA), a method that allows for multiple inserts to be assembled into a vector backbone in one reaction by taking advantage of Type II restriction endonucleases that cleave DNA outside of the recognition sequence, leaving unique overhangs that allows for orderly assembly of multiple fragments [22, 23].

3.1.1 Preparation of Cas9 Plasmid and Guide RNA (gRNA) Backbone

1. Thaw plasmids: gRNA plasmid P396, Cas9 plasmid, and FP plasmid.
2. Add 1 mL of 100 mg/mL ampicillin to 1 L of autoclaved lysogeny broth (LB).
3. Prepare two flasks of 200 mL ampicillin LB (or other appropriate antibiotic) in 1 L Erlenmeyer flasks and inoculate one with the Cas9 plasmid and the other with the FP plasmid.

4. Inoculate 4 mL ampicillin LB in 14 mL round bottom tube with the gRNA backbone plasmid.
5. Incubate both at 37 °C in a large shaking culture incubator overnight (approx. 16–18 h).
6. Use a Midiprep kit to isolate the Cas9 and FP plasmids from the 1 L cultures.
7. Use a Miniprep kit to isolate the gRNA backbone plasmid from the 14 mL culture.

3.1.2 Designing and Cloning Guide RNA (gRNA)

1. Import the genomic sequence of the gene into software that enables genome sequence annotation, such as to Benchling in this example. In Benchling, click the add (+) button at the top of the left-hand menu. Hover over "DNA sequence" and select "import DNA sequence" option. Go to "import from database" tab. In search bar, enter gene name, NCBI accession number, ENSEMBL ID, etc., enter the genome to search, and click "search". Verify that the entry found is the correct entry. Select folder and click "import" (Fig. 1).
2. Find the exon that has the start codon with the first annotated amino acid (Methionine) and take note of the location for future reference.
3. Go to indelphi.giffordlab.mit.edu or equivalent in silico gRNA design webtool and click on "Gene Mode" tab (Fig. 2a).
4. Choose your reference genome species of choice, type your gene of interest into the search bar, and choose cell type (*see*

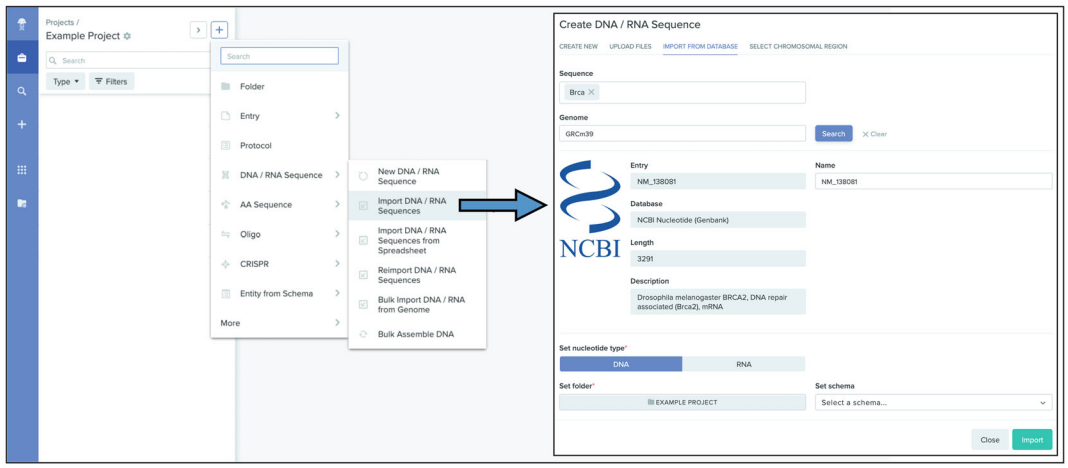

Fig. 1 Importing gene of interest into Benchling. Using Benchling, import the sequence of your gene of interest by clicking the plus sign button at the top right of the menu, when in a project folder. Click "import DNA/RNA sequence". Go to the "import from database" tab, and search for the gene name or NCBI accession name. Then click "import"

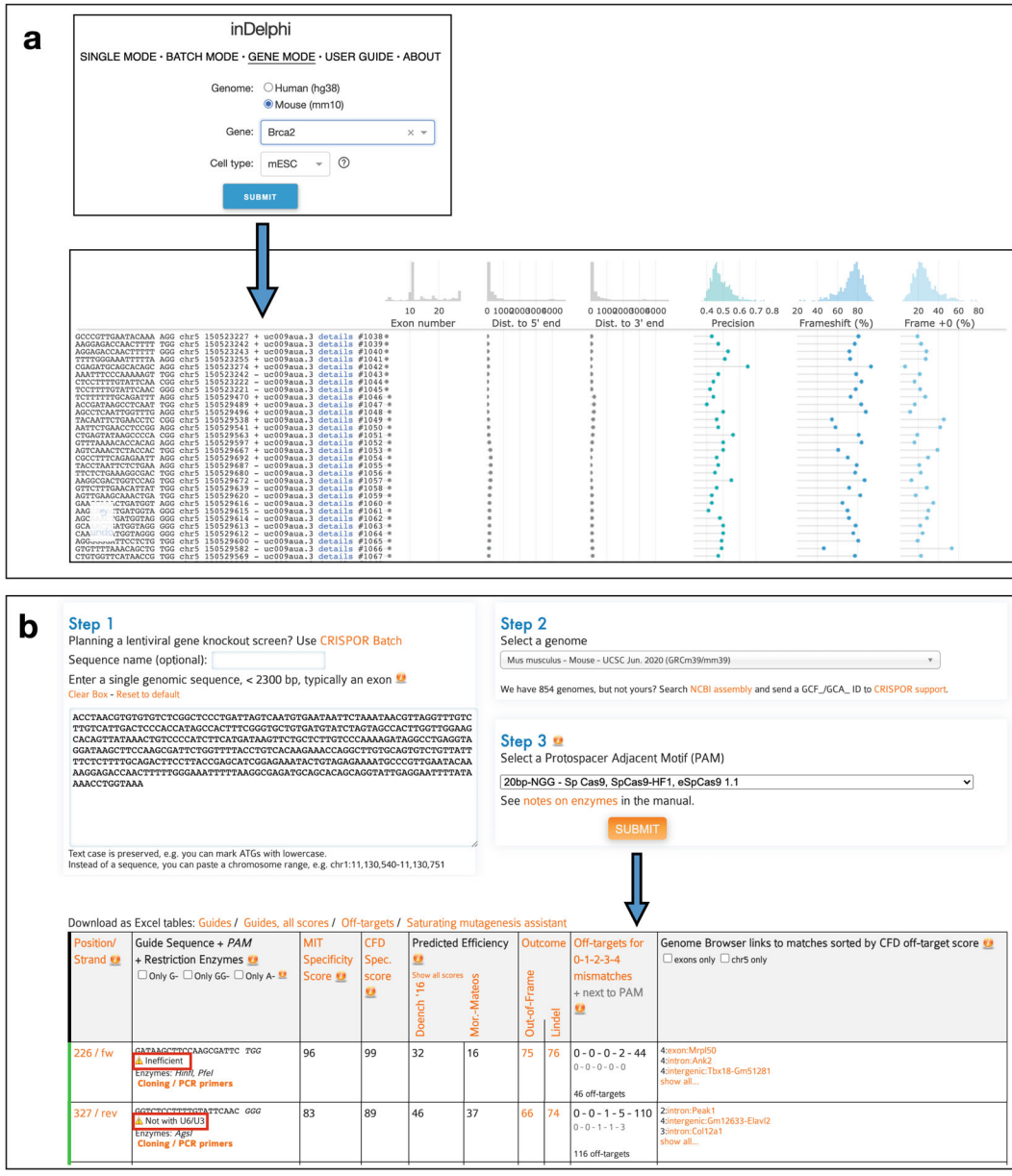

Fig. 2 Designing gRNAs using inDelphi and CRISPOR. (**a**) Go to the "Gene Mode" tape on inDelphi. Search for the gene of interest in the correct genome. Click "submit". The following page will show a large list of potential gRNAs. Take note of the sequence, precision rate, and frameshift percent. (**b**) Once you have identified a few gRNA sequences from inDelphi, double check them on CRISPOR. Enter the exon sequence from your gene, and select the genome. Click "submit". The following page will show a list of gRNAs. Search for the ones you found on inDelphi to get the predicted efficiency, off-target sites, and any restrictions. Some restrictions, like inefficient and not with U6/U3, are shown in red boxes

Note 1). Click submit. The results of the search will be a large list of potential gRNA sequences.

5. Choose 2–3 different gRNA sequences using the following criteria: (i) choose gRNAs that are located in an early exon that contains parts of the ORF, i.e., target the first exons after the start codon (*see* **Note 2**), (ii) choose gRNAs with high precision, meaning the target sequence is singular in the genome and the next closest sequence differs in as many nucleotide positions as possible, and (iii) choose gRNAs with high frameshift occurrence and low frame +0 occurrence (assessed in InDelphi).
6. Take note of the gRNA sequences that you have chosen by typing the sequence into an entry or annotating the genomic sequence on Benchling.
7. Go to CRISPOR.tefor.net to check cutting efficiency, off-targets, and specific cloning requirements for the gRNAs chosen from **step 5**. Enter the sequence of one of the exons where a gRNA targets in the box to the left (Fig. 2b).
8. Select the mouse genome and select the NGG protospacer adjacent motif (PAM) used for spCas9. Click submit.
9. Search for your chosen gRNA sequence and review any cloning requirements (*see* **Note 3**), predicted off-target sites (*see* **Note 4**), and predicted efficiency (*see* **Note 5**).
10. Once your gRNA selections have been checked using inDelphi and CRISPOR, you can finalize your selections and annotate your sequence on Benchling.
11. Copy the DNA sequence of your gRNA from the gene sequence and add to a blank entry and label as "sense strand" or "S/S". This is the forward sequence to your gRNA.
12. Go back to the annotated gRNA on the gene sequence and copy the reverse complement. To do this on Benchling, right click, hover over "copy special" and pick "Reverse Complement" to copy the reverse complement of the gRNA sequence. Add this to the same blank entry and label as "antisense strand" or "A/S". This is the reverse sequence to your gRNA.
13. Add BbsI restriction enzyme sticky-end overhangs onto each sequence. Add CACC to the beginning of the "sense strand" sequence and add CAAA to the beginning of the "antisense strand" sequence (*see* **Note 6**).
14. Order these gRNAs as custom DNA oligos from a nucleic acid synthesis provider (e.g., Integrated DNA Technologies (IDT)).
15. Once you have received the ordered oligos, dilute oligos to stock solutions of 100 μM using nuclease-free water.

16. Anneal oligos together by adding 1 μL of the 100 μM (approximately 750 ng) sense oligo stock solution, 1 μL of the 100 μM anti-sense oligo stock solution, and 7 μL nuclease-free water into a clean PCR tube. Mix gently.
17. Place the tube in a thermocycler and use the following program for slow annealing of the oligos:

    95 °C for 5 min

    Ramp down 0.1 °C per second to 25 °C

    Keep at 4–12 °C until next step
18. Prepare the Golden Gate Assembly (GGA) reaction by combining 2 μL annealed oligos from **step 17**, 20 Units BbsI, 1000 Units T4 Ligase, 100 ng gRNA plasmid backbone prepped in Subheading 3.1.1 (P396), 2 μL ligation buffer (Promega), and nuclease-free water up to 20 μL total volume in a clean PCR tube.
19. Run the GGA by putting the tube in the thermocycler using the following program:

    37 °C for 30 min

    37 °C for 3 min

    16 °C for 5 min

    Repeat **steps 2** & **3**, 15 times

    37 °C for 30 min

    65 °C for 20 min

    Hold at 12 °C
20. Transform ~2–2.5 μL GGA solution into competent bacteria (e.g., TOP10). Place the cells on ice for 30 min, heat shock bacteria by placing the tube in a 42 °C heat block for 30 s, then placing the tube back on ice for 2 min. Then add approximately 200 μL of SOC media to the tube, and place in a heat block at 37 °C for 30–60 min shaking at 650 rpm.
21. Plate on a carbenicillin or ampicillin plate using glass beads. Place the plate in an incubator at 37 °C overnight.
22. The next day, pick 2–3 different colonies from the plate to grow in small cultures overnight. To do this, take a sterile/autoclaved pipette tip or toothpick and pick up one bacterial colony (*see* **Note 7**), touch a separate labeled carbenicillin plate to document the specific colony chosen, and then swirl the toothpick into 4 mL of ampicillin lysogeny broth (LB) in a capped 14 mL tube. Place the touch plate in the incubator at 37 °C and the LB culture in a shaking incubator set to 37 °C, 230 rpm both for overnight.
23. The next day, take the cultures out of the shaking incubator and isolate the plasmid DNA using a miniprep kit.

24. Verify that the chosen colonies contain the correct plasmids via diagnostic restriction enzyme digests using the BbsI enzyme to confirm cloning sites have been removed (optional) and sequencing.
25. Inoculate a 200 mL LB ampicillin culture of the sequence-verified colony from the touch plate in a 1 L Erlenmeyer flask and place it the shaking incubator at 37 °C overnight.
26. The next day, take the culture out of the shaking incubator and isolate the plasmid DNA using a midiprep kit and check concentration and purity on a spectrophotometer (e.g., NanoDrop).
27. Ethanol-precipitate the gRNA and Cas9 plasmid DNA (from Subheading 3.1.1) by adding 600 μL 100% ethanol and 60 μL of 3 M Sodium Acetate to the eluted DNA, then centrifuge at 15,000 g, or max speed on a tabletop centrifuge for 20 min at 4 °C. Remove the supernatant from the white DNA pellet without disturbing the pellet. Let pellet air dry for 15 min, or put on a shaking heat block at 37 °C until dry.
28. Add enough elution buffer to make the final concentration of plasmid DNA around 4 μg/μL and confirm concentration using a spectrophotometer (e.g., NanoDrop).

3.2 In Utero Electroporation

In the following steps, we outline how to perform in utero electroporation in the developing mouse brain. This technique can also be performed on rats, with a few minor changes to the age of the embryo, equipment sizes, and electroporation parameters. This technique allows one to introduce plasmid DNA into the progenitor cells lining the ventricles of the developing rodent brain, and therefore tag specific populations of neurons. This chapter will go through the steps to target layer 2/3 intracortical neurons of the somatosensory cortex and pyramidal neurons in CA1/CA3 of the hippocampus.

3.2.1 Surgery Preparation

Mouse strain: Timed-pregnant CD1 female mice from Charles River Laboratories were used for their robust maternal behavior. However, it is an outbred strain, with less reliable DNA database sequence consistency. Therefore, it is recommended that gDNA of the gene of interest be sequenced prior to designing gRNAs.

1. Either breed or order timed-pregnant CD1 mice from Charles River Laboratories to be embryonic day 15 (E15) for layer 2/3 cortex or E16 for hippocampus electroporation (*see* **Note 8**).
2. Turn on heat pad to 37 °C, heat block to 40 °C, and electroporator.
3. Place two 50 mL conical tubes of sterile 1× PBS into the heat block to warm up for at least 15 min before the start of surgery.

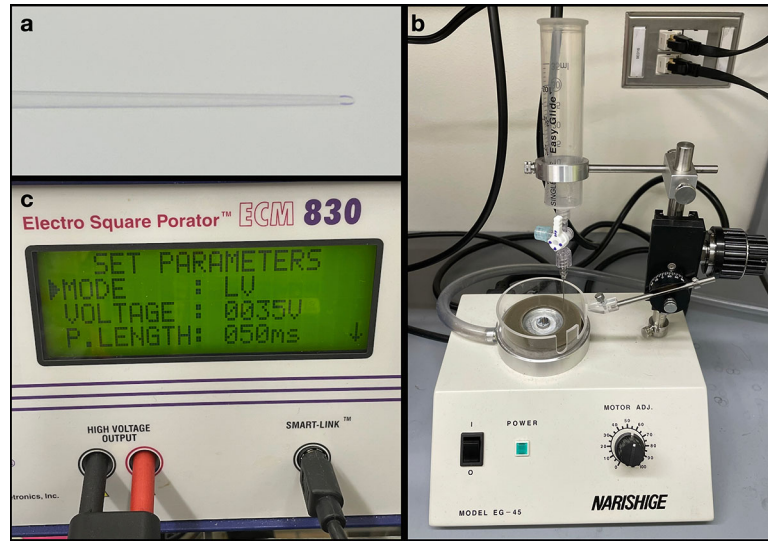

Fig. 3 Surgery preparations. (**a**) Glass capillary tubes were pulled to a point using a Narishige pipet puller. They were then beveled at a 30° angle with a larger opening. (**b**) Narishige rotating beveler set to 30° angle with a syringe above the diamond bit to drip distilled water throughout the grinding. (**c**) Electroporator has been set to the correct settings for cortical electroporation

4. Pull and bevel several glass capillary tubes to create glass micropipettes that have a rounded sharp point with a small opening at the end. These will be used to inject your DNA into the embryonic brains (*see* **Note 9**).
5. Use a beveler with a rotating diamond wheel and an angle-adjusting micropipette holder (Fig. 3b). Bevel the tip at a 30–35° angle with constant water flow to prevent chipping with wheel rotation at approx. 500–1200 rpm. Check under a stereoscope to make sure none have been broken or blocked (Fig. 3a).
6. Prepare a plasmid DNA mix for the surgery. Combine equal amounts of gRNA, Cas9, and FP plasmids to a total concentration of 4 μg/μL in elution buffer from the plasmid prep kit. Dilute fast green 1:10 in DNA mix. Make 10–20 μL total per pregnant animal.
7. Plug the tweezertrodes into the electroporator and set at the following parameters (Fig. 3c):

   Pulse Voltage: 35 V

   Pulse Length: 50 ms

   Number of Pulses: 4

   Interval Between Pulses: 1 s

8. Gather the sterile bench pad, sterile gauze, eye lubricant, sterile cotton tip applicators, alcohol antiseptic pads, parafilm, hair removal cream, sutures, beveled capillaries, aspirator, analgesic in 1/2 cc syringe with 27 G needle, and sterilized surgical tools.

3.2.2 In Utero Electroporation Surgery

All animals were treated in accordance with the regulations and guidelines of the Institutional Care and Use Committee of the University of Maryland School of Medicine and the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23) revised 1996.

1. Prepare the mouse for surgery by placing it in the induction chamber at 3% isoflurane with 100% oxygen as the carrier until the animal's breathing has slowed to approximately 1 breath per second (approx. 3–5 min).
2. Remove the mouse from the induction chamber and place head in the nose cone with the animal on its stomach.
3. Give analgesic (e.g., Buprenorphine) and put eye lubricant on the eyes. Add more eye lubricant every 20 min during the surgery.
4. Flip the mouse onto its back and replace its head in the nose cone.
5. Prep the surgery site by swiping an alcohol wipe across the abdominal area.
6. Add a small amount of hair removal cream to the abdominal area, and create a small oval area in the lower abdomen (Fig. 4a).
7. Use a cotton tip applicator to spread the cream around the area until the hair is lifted.
8. Use 1× PBS and a Kim wipe to remove the excess hair and hair removal cream (*see* **Note 10**).
9. Once all of the hair is removed, apply a sterile alcohol pad and Betadine surgical scrub to sterilize the surgical area, repeating 3 times. Remove any residual Betadine scrub with sterile 1× PBS.
10. Cut a small hole in the sterile gauze that matches the size of the hairless area of the abdomen. Place the cut gauze around the area, and wet the edges with warm 1× PBS (*see* **Note 11**).
11. Verify that the mouse is under deep anesthesia with a toe pinch.
12. Make a 1 inch incision in the skin along the midline of the lower abdomen (*see* **Note 12**).
13. Use microscissors to cut away the connective tissue that connects the skin layer and the muscle layer below. This will aid in suturing.

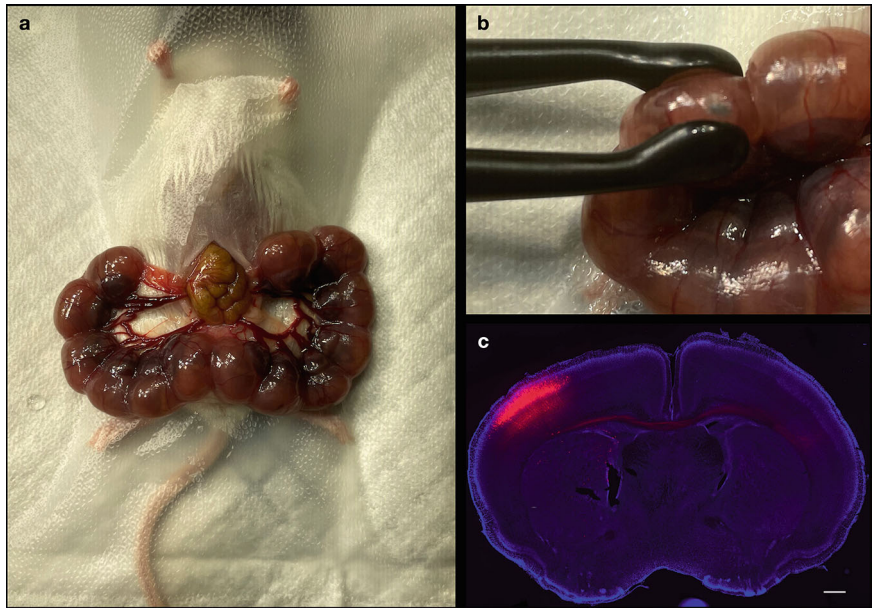

Fig. 4 In utero electroporation. (**a**) The uterine horn was exposed following a laparotomy. (**b**) Plasmid mix containing green dye was injected into the lateral ventricle of an E15 pup and tweezertrodes were placed on either side of the embryo's head, with the positive electrode on the same side as the injection, for a cortical electroporation. (**c**) A coronal section showing a positive cortical electroporation. Upper layer pyramidal neurons expressing red fluorescent protein (red) largely coexpress Cas9 and gRNAs to induce knockout of a gene. Their cell bodies are seen at the focus of the electroporated area (inset close up), while their axons can be seen projecting across the corpus callosum. Brain was perfused, sectioned at 80 μm, stained for RFP, and mounted using Fluoromount with DAPI. Scale bar 500 μm, inset 100 μm

14. Create a similar size incision along the midline of the muscle layer. Be careful not to cut too deep as the internal organs are pressed up against the muscle layer. Create a small hole first to lift the muscle away from the organs.
15. Now that the abdominal cavity has been opened, it is imperative to add warm sterile 1× PBS to the cavity every 2–3 min, or sooner if it looks dry.
16. Prepare your micropipette by placing the blunt end into the aspirator adapter. Aspirate any water that is left in your glass micropipette tip from beveling to prevent dilution of your DNA.
17. Pipette ~5 μL of your DNA mix onto a piece of parafilm.
18. Place the tip of the glass micropipette in the drop of DNA at a horizontal angle to prevent breaking the tip against the parafilm.
19. Suck up as much DNA as you can without introducing any air bubbles. Place the aspirator and needle safely aside.

20. With the blunt forceps, carefully grab at the node between two embryos and pull to remove the uterine horn from the abdominal cavity. Do not pull hard as you do not want to detach the uterine horn from the abdominal wall.
21. Pull on one side until you see a small knot of muscle that is attached to the abdominal wall. Repeat on the other side. Be cautious of the uterus that connects both sides of the uterine horn in the middle (Fig. 4a).
22. Wet with warm 1× PBS.
23. Wearing sterile gloves and using your fingers, carefully pick up one of the embryos in its amniotic sac. Note the orientation of the embryo. If you cannot see the top of the head, carefully palpate the embryo to rotate it in the sac. Be careful not to squeeze too hard as you might rupture the amniotic sac.
24. Once you have a clear view of the top of the head, locate the midline of the brain. On either side of this line are the lateral ventricles, often seen as dark shadows.
25. While holding the embryo with one hand, pick up your aspirator and glass micropipette. Poke the glass micropipette through the amniotic sac (*see* **Note 13**) and into the embryonic brain. Do not poke far into the brain, as the lateral ventricle is very shallow. Once you pierce through the edge of the brain, you are most likely in the ventricle.
26. Once you think your glass micropipette is in the lateral ventricle, blow through the aspirator at a slow steady pace. You should see blue dye filling up the ventricle (*see* **Note 14**). Fill the ventricle until you see a bright blue spot, this is approximately 0.75–1 μL (Fig. 4b).
27. Once you have filled the ventricle, while holding the embryo with one hand, put aside your aspirator and glass micropipette, and pick up the tweezertrode. To electroporate the cortex, place the positive electrode on the same side as the injection, and the negative electrode on the other side of the head. Both should be around the ear area (Fig. 4b). Lightly squeeze the head in between the tweezertrode without rupturing the amniotic sac (*see* **Note 15**). Once you are in the right position, start the electroporator and wait until all pulses are compete.
28. Remove the tweezertrodes, let go of the embryo, and wet with warm 1× PBS. To electroporate the hippocampus, reverse the positive and negative electrode positions.
29. Repeat **steps 23–28** on 3–6 other embryos, depending how many embryos are available (*see* **Note 16**).
30. Once you have completed all of your electroporations, carefully push the uterine horn back into the abdominal cavity using forceps to hold the skin/muscle back and your fingers to gently

push. Do not push with too much pressure as you can rupture the amniotic sac.

31. Once all embryos are back inside the abdominal cavity, fill it up with warm 1× PBS.
32. Suture the muscle layer and skin layer separately, using a continuous stitch.
33. Sterilize the incision with an alcohol pad.
34. Place the mouse back in its home cage, on its back under a red heat lamp.
35. Monitor the mouse for approximately 1 h to make sure it is not in pain and can move freely.
36. Continue to monitor the mouse daily until she gives birth, around E19–20.
37. Once the pups are born, screen them under a fluorescence stereoscope (*see* **Note 17**) non-invasively (*see* **Note 18**) by gently holding them at the focal distance and observing fluorescent protein signal to identify which pups have been successfully electroporated (*see* **Note 19**).

4 Notes

1. For rodent in utero electroporation, we will be using the mouse genome. If your cell type of interest is not listed in the menu, choose mESC if your cell type has no expected defects in DNA repair.
2. To check where the gRNA will cut, copy the gRNA sequence and search your gene sequence imported into Benchling. You can also annotate the Benchling sequence to save the gRNA sequence, by using the "Annotations" button on the right-hand menu of the sequence page.
3. Some gRNAs will indicate that they are inefficient or require a different promoter other than U6/U3. Choose gRNAs that do not have these warnings.
4. Choose gRNAs that do not have any off-targets with mismatches of 2 or less. Any gRNAs that have 3 or more mismatches with off-target sites are likely to be precise. Also check the MIT specificity score that is based on Hsu and colleagues [24]. The higher the score, the less likely there will be off-target effects.
5. The higher the efficiency score, the more likely there will be cleavage at that position in the genome. The predicted efficiency score is based off of two studies, the first being by Doench and colleagues [25] which uses Azimuth scores that

are based on data from cells, and recommended for gRNAs that use U6 as a promoter. The second score is from Moreno-Mateos and colleagues [26] which uses CrisprScan scores that are based on data from zebrafish embryos, and recommended for gRNAs that use T7 as a promoter.

6. If the gRNA sequence ends in T or A, add an additional C or G to increase cloning efficiency. Remember to add the corresponding G or C on the reverse strand to complement.
7. Be sure not to pick up more than one colony at a time, as this may result in a mixture of different plasmids. If the plate does not have singular colonies, take a sterile or autoclaved pipette tip or toothpick and streak it out on a separate carbenicillin plate to get single colonies. Then proceed to the next step.
8. Embryonic day zero is the morning that the vaginal plug is observed in mated female rodents. Also referred to as days post coitum (dpc).
9. Make sure that the opening is wide enough to be able to eject viscous liquid like your DNA mix, but not too wide as to damage the amniotic sac or brain of the embryo. We use an opening in the range of 50 μm. Also make sure that there are no chips or debris on the tip of the needle. Make 3–5 in total to have extra during the surgery in case you accidentally break one.
10. Be sure to avoid the ring of nipples around the abdomen, as you do not want to irritate them.
11. Wetting the surface of the gauze gives the embryos a warm and moist landing spot when we remove the uterine horn from the abdominal cavity.
12. Do not cut too low on the abdomen, as there is a large blood vessel that, if cut, can lead to death via exsanguination.
13. Do not hold the amniotic sac too tightly or when you poke your needle through, it will rupture.
14. Hold the needle as you would a pencil for good control of its location. If you do not see blue dye come out of the needle during the injection, take the needle out and try to blow out in the air. If nothing comes out, your needle is most likely blocked by brain matter, and you need to prepare a new one. This also means that you most likely inserted the needle too far into the embryonic brain.
15. You should see bubbles forming at the negative electrode and see the embryo twitch at each pulse. This shows that there was a good electrical field.
16. Do not electroporate the embryos closest to the abdominal wall or uterus on either side. The electrical pulses could cause

contractions and miscarriage. Do not electroporate more than 8 embryos in one dame, this could also cause miscarriages.

17. You can screen pups using a fluorescence stereoscope that has long working distance or other macroscopic fluorescence options like blue LED flashlight and orange filter glasses for GFP or corresponding LED and filter glasses got other FPs.
18. When using bicistronic plasmids that co-express Cas9 and FP, fluorescence is typically too weak to screen pups non-invasively. This may be due to reduced FP expression from the large bicistronic mRNA. To facilitate screening, we suggest to have Cas9 and FP on separate plasmids that are mixed and co-electroporated. However, if screening is not a problem, you may choose a single plasmid expressing both Cas9, an FP, as well as the gRNA cassette driven by a Pol III promoter like U6.
19. Some of the electroporated pups may not be born, as they may be resorbed before birth, or be stillborn. If this is the case, try to be gentler with your injection in your next surgery as you may have caused damage. Do not be discouraged, this surgery takes a lot of practice and patience.

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