STAR Protocols

Protocol

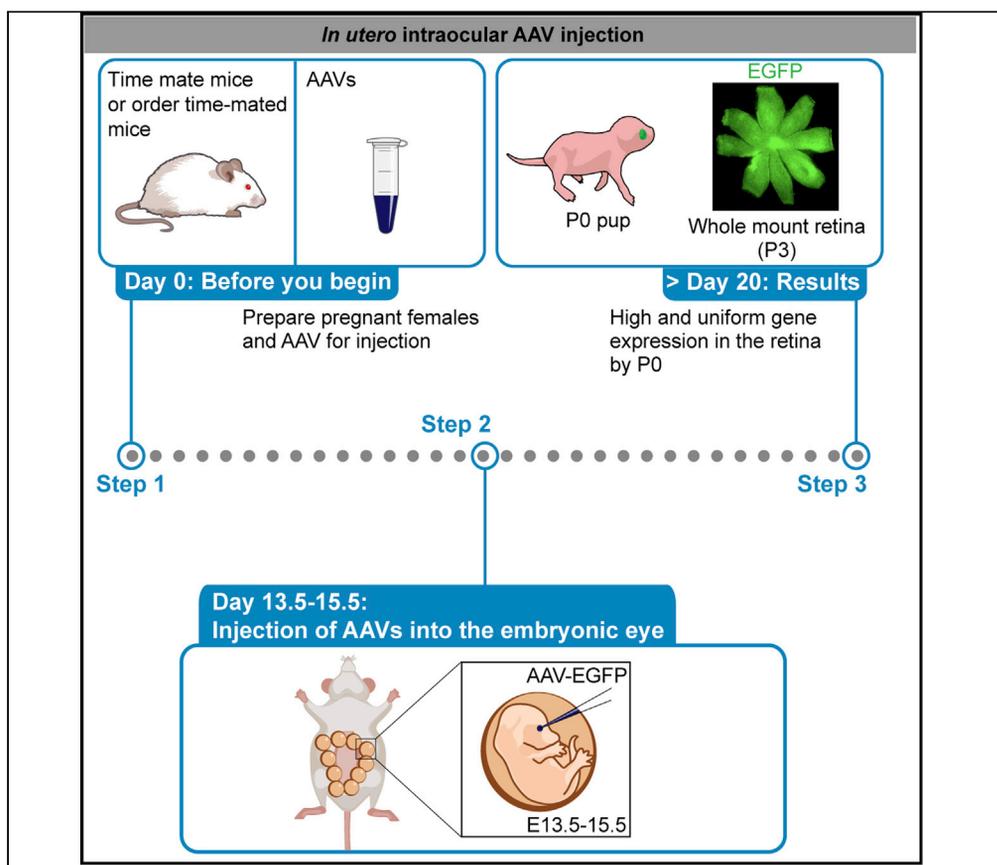# *In utero* intraocular AAV injection for early gene expression in the developing rodent retina

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Highlights

In utero intraocular
AAV injection to
express genes in the
developing mouse
retina

High and uniform
expression of genes-
of-interest in the
newborn retina

Ability to
independently
manipulate each eye

Suitable for
examining early visual
system development

The visual system is the best system to study activity-dependent sensory circuit development. The connections from the retina to the dorsal lateral geniculate nucleus, the retinogeniculate connections, undergo extensive remodeling during early postnatal life. Thus, techniques that allow the expression of transgenes early in the developing retina are essential to study visual system development. Here, we describe a protocol to express genes-of-interest in the developing mouse retina via *in utero* intraocular adeno-associated virus injections.

Protocol

In utero intraocular AAV injection for early gene expression in the developing rodent retina

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SUMMARY

The visual system is the best system to study activity-dependent sensory circuit development. The connections from the retina to the dorsal lateral geniculate nucleus, the retinogeniculate connections, undergo extensive remodeling during early postnatal life. Thus, techniques that allow the expression of transgenes early in the developing retina are essential to study visual system development. Here, we describe a protocol to express genes-of-interest in the developing mouse retina via *in utero* intraocular adeno-associated virus injections.

For complete details on the use and execution of this protocol, please refer to Yasuda et al. (2021).

BEFORE YOU BEGIN

Preparation of pregnant mice

⏱ Timing: [13–16 days]

1. Time mate mice or purchase time-mated females such that embryos will be embryonic day 13.5–15.5 (E13.5–E15.5) on the day of surgery.

   Note: Embryos can be injected up to E15.5 to ensure high and uniform viral expression in the newborn retina. However, we normally use E13.5 embryos. The eyes of older embryos (e.g., E15.5) are easier to inject, but it is more difficult to return the uterus to the abdominal cavity when embryos are older. Mice younger than E13.5 are smaller in size, thereby making the identification of the embryonic eye more difficult.

   Note: In Yasuda et al. (2021), time-mated female mice were purchased from Charles River Laboratories. Upon arrival at Boston Children's Hospital, pregnant female mice are singly housed and allowed to acclimate for at least 3 days prior to surgery. All animal care and experiments were performed in accordance with the institutional guidelines approved by the Institutional Animal Care and Use Committees at Boston Children's Hospital.

Preparation of AAV

⏱ Timing: [prior to surgery]

2. Prepare AAV (serotype: DJ; titer: ~1×10^12 gc/mL) virus encoding the gene-of-interest.
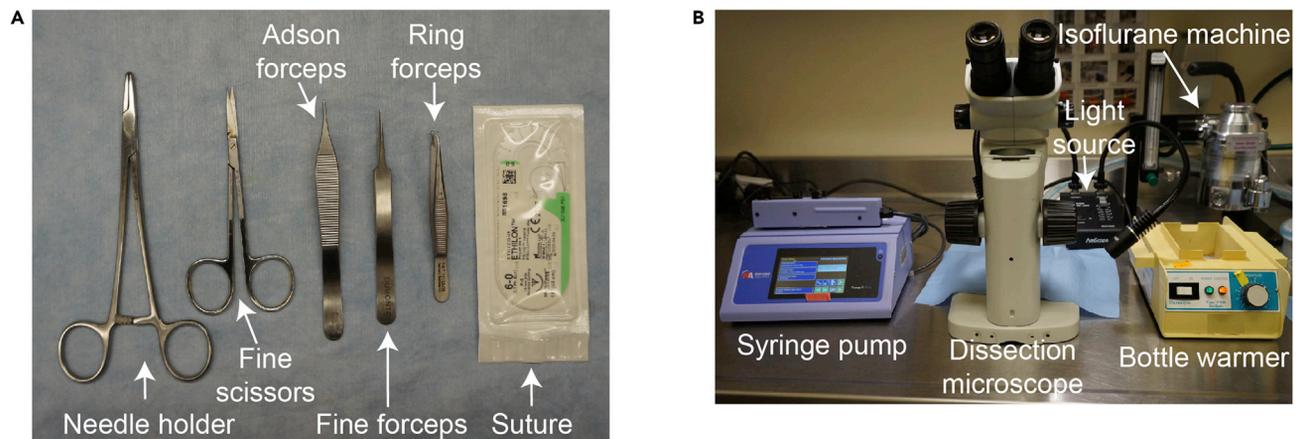
Figure 1. Essential surgical tools and equipment for *in utero* intraocular eye injections
(A) Image of surgical tools.
(B) Image of the equipment needed.

3. Mix in the fast green dye such that the final concentration of dye is 0.1%.

   Note: We use the AAV-DJ serotype because of its rapid expression (Lakhan et al., 2015). See limitations below for the discussion on AAV serotypes. In Yasuda et al. (2021), AAVs were generated by the Boston Children's Hospital viral core and used in accordance with the Boston Children's Hospital Institutional Biosafety Committee.

Preparation of surgical instruments

⊙ Timing: [1–2 days]

4. Prepare glass micropipettes for injections.
   a. Pull borosilicate glass capillaries (1.0 mm outer diameter; 0.75 mm inner diameter; TW100-4; World Precision Instruments LLC., USA). We use a Flaming/Brown micropipette puller (Model P-1000; Sutter Instrument Co., USA). Micropipettes are pulled with the following setting: HEAT: Ramp+10; PULL: 30; VELOCITY: 120; TIME: 200; PRESSURE: 200.
   b. Break the tip of the glass microcapillary with fine forceps to create a sharp angle tip with an outer diameter of 20–50 μm.

   Note: A good microcapillary pipette is important for this protocol. We recommend preparing ~10 micropipettes of different tip sizes ranging from 20–50 μm. When choosing the micropipette, begin with the narrowest one. If loading the viral solution is difficult, use the next narrowest micropipette and so on until the virus solution loads smoothly. Since the thickness of the uterine wall varies between each pregnant female, narrow micropipettes may break during injection if the uterine wall is thick. If so, size up.

5. Sterilize all surgical instruments (Figure 1A) and glass micropipettes via autoclave or ethylene oxide gas.
6. Ensure all drugs and consumables (sterile gloves, drapes, and gauzes) are available and unexpired.
7. Prepare surgical equipment (Figure 1B) and clean by wiping with disinfectant wipes (Peroxigard).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
AAV-DJ-CAG-EGFP	Boston Children's Hospital Viral Core	N/A
Chemicals, peptides, and recombinant proteins		
Isoflurane	Patterson Veterinary	NDC: 14043-704-05
Loxicom (Meloxicam) injection 5 mg/mL	Norbrook	NDC: 55529-040-10
Povidone-iodine (Betadine) scrub 7.5%	Purdue Products	NDC: 67618-154-16
OptixCare eye lube	OptixCare	N/A
0.9% Sodium chloride (saline)	Hospira	NDC: 0409-4888-02
Cholera toxin subunit B, CF488 dye conjugates (CTB488)	Biotium	Cat. No. 00070
Fast green FCF	Sigma-Aldrich	Cat. No. F7252
Peroxigard wipes	Virox Technologies	N/A
Experimental models: organisms/strains		
Mouse: CD1 (pregnant females)	Charles River	Cat. No. 022
Mouse: C57BL/6J (pregnant females)	Jackson Laboratories	Stock No. 000664; RRID: IMSR_JAX:000664
Other		
Pump 11 Elite Nanomite	Harvard Apparatus	Cat. No. 70-4507
Ring forceps	Fine Science Tools	Cat. No. 11106-9
Dissecting microscope	Olympus	N/A
LED dual gooseneck illuminator	Unitron	Cat. No. 15857
Glass capillaries	World Precision Instruments	Cat. No. TW100-4
Micropipette puller	Sutter Instrument	Cat. No. P-1000
Needle holder	Fine Science Tools	Cat. No. 12004-16
Suture Ethilon 18" P-3	Medline Industries	Cat. No. ETH1698G
Fine scissors	Fine Science Tools	Cat. No. 14568-12
Fine forceps	Fine Science Tools	Cat. No. 11251-10
Adson forceps	Fine Science Tools	Cat. No. 11018-12
GFP goggles	BLS	Cat. No. FHS/EF-2G2
Nosecone	VetEquip	Cat. No. 921411 and 921609

MATERIALS AND EQUIPMENT

All materials, equipment and their sources are listed in the text and in the key resources table.

STEP-BY-STEP METHOD DETAILS
Preparation of mice for surgery

⏲ Timing: [30–40 min]

1. Place a bottle of sterile saline on the bottle warmer and allow the saline to reach 37°C.
2. Weigh the mouse and administer analgesic. In Yasuda et al. (2021), 5–10 mg/kg of Meloxicam was injected subcutaneously.

   Alternatives: Other analgesics approved by the respective institutional animal care and use committees may be used for this protocol.

   Note: Intraperitoneal injections into pregnant females should be avoided as they may harm the embryos. Additionally, this protocol requires the opening up of the abdominal cavity. This may render the analgesic less effective.
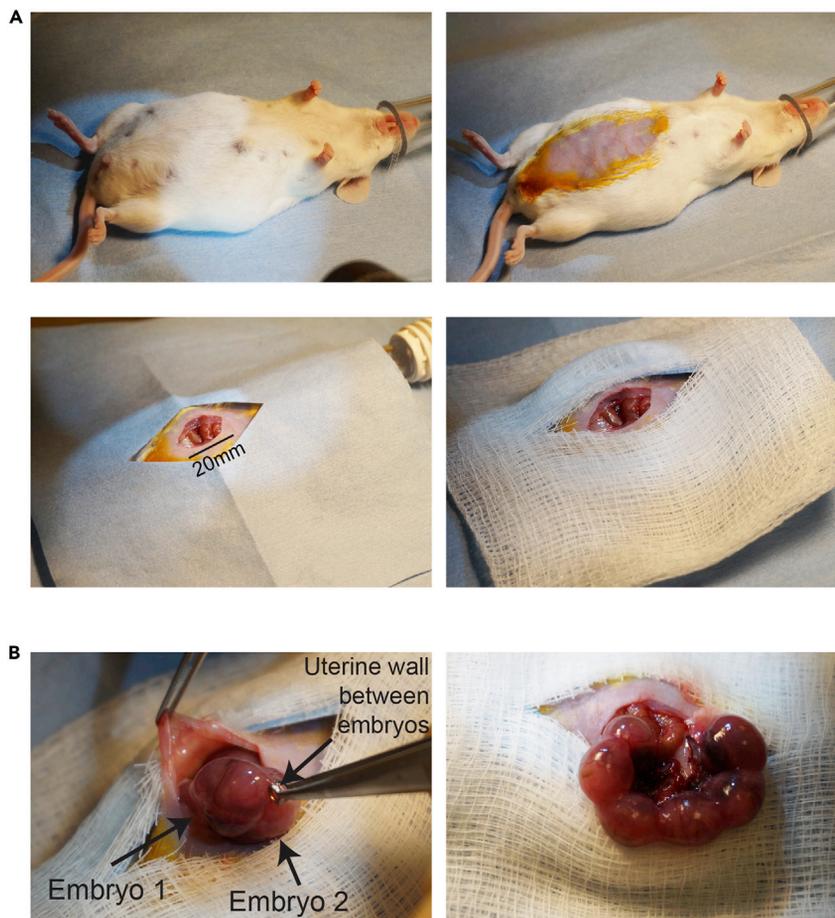
Figure 2. Preparation of pregnant female mouse for surgery and exposure of embryos

(A) Method of preparing the pregnant female for surgery. Place an anesthetized pregnant female on the operating stage. Shave all fur from the abdomen and sterilize abdomen by scrubbing with alternating Povidone-iodine (Betadine) and 70% ethanol wipes three times. Cover the mouse with sterile surgical drapes. Make a 20–30 mm long incision of the skin followed by the abdominal wall along the midline. Cover the area with sterile gauze.

(B) Method of exposing embryos. Expose the embryos by gently lifting the abdominal wall with Adson forceps and pulling the uterus out by holding the uterine wall between embryos using ring forceps.

3. Anesthetize a pregnant mouse in an induction chamber with 1 l/min flow rate of 3% Isoflurane until the animal is anesthetized completely.

   Note: Check that the mouse is fully anesthetized by monitoring breathing rate and assessing the toe pinch reflex.

4. Apply eye lubricant to the eyes to prevent dry eyes.
5. Place the mouse on a surgical drape-lined operating stage under a dissection microscope (Figure 2A).
6. Attach a nose cone (VetEquip) to the mouse's nose. Adjust the isoflurane settings such that the mouse receives 2–3% isoflurane at a rate of 0.5–1 l/min.

   ⚠ CRITICAL: Monitor breathing rate (about 50–70 breaths per minute) (Ewald et al., 2011) during the entire procedure to avoid isoflurane overdose.

7. Remove all fur on the abdomen with a razor (Figure 2A).
8. Scrub the abdominal skin with Povidone-iodine (Betadine) followed by 70% ethanol. Repeat three times.

9. Cover the mouse with a sterile drape that has a 40 mm × 40 mm opening centered over the abdomen (Figure 2A).

Exposure of embryos

Ⓣ Timing: [5 min]

10. Cut the skin from anterior to posterior along the midline (20–30 mm), then cut the abdominal wall (muscles and peritoneum; 20–30 mm) with fine scissors (Figure 2A).
11. Cover the abdomen with sterile gauze that has a 40 mm-long cut at the center. Moisten the gauze around the cut site with saline.
12. Lift the abdominal wall with Adson forceps and gently pull out the embryo-containing uterus, starting with the embryo nearest to the opening. Hold the uterine wall between two embryos with ring forceps and gently pull (Figure 2B). When two embryos are pulled out, hold on to the uterine wall between the next two embryos and gently pull another embryo out. Repeat this until all embryos are exposed.

    Note: Keep the uterus wet throughout the procedure by gently pipetting warm sterile saline onto the uterus. Avoid damaging the placenta and blood vessels with forceps.

Intraocular AAV injection

Ⓣ Timing: [20–25 min]

13. Load (up to 0.5 μL) virus solution colored with fast green (0.1%) to micropipette.

    Note: For high levels of viral transduction, we injected 0.2 μL of a high titer AAV (1×10^{12} gc/mL). If the viral titer is lower or multiple AAVs need to be injected, a larger volume, up to 0.5 μL of viral solution, may be injected per eye. If the injected volume exceeds 0.5 μL, the viral solution may leak from the injected eye.

    Note: This protocol may be used to inject only one eye or both eyes.

14. Adjust the embryo within the uterine wall by gently pushing the back of the embryo until the eye is facing up. Gently stabilize the embryo by holding the uterine wall just under the embryo's head with ring forceps. Maintain the width between the ring forceps' tips at about 50–70% the width of the embryo (Figures 3A and 3B)
15. Gently push the embryo up from under the embryo's head with the ring forceps (maintaining the width between the tips) to move the embryo as close to the uterine wall as possible (Figures 3A and 3B).

    ⚠ CRITICAL: Do not hold or damage the placenta as this would induce a miscarriage.

16. Locate an eye under the dissection microscope (Figure 3B).

    Note: Ensure aseptic technique. To avoid contaminating the sterile gloves, we use sterilized aluminum foil or sterile drapes to handle any non-sterile surfaces.

17. Adjust the angle of the fiber optic light source cables to make the embryo's eye more visible.

    Note: Usually, light directly onto the side of the embryo's body gives better visibility of the eye (Figures 3A and 3C). The angle of the light source will need to be adjusted for each embryo.
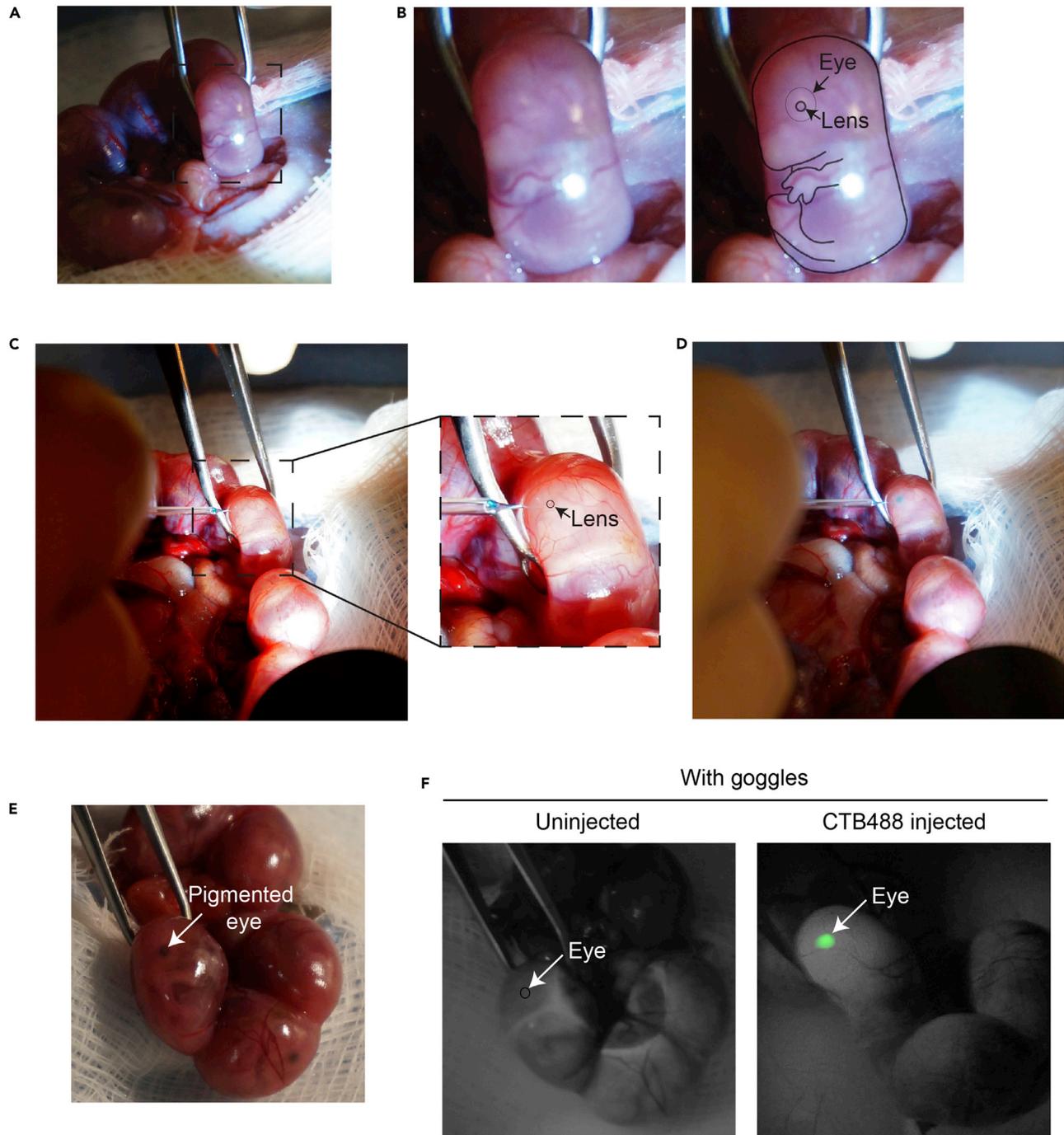
Figure 3. Identification of the embryonic eye and AAV injection

(A) Image shows an exposed CD1 embryo positioned for injection. Hold the embryo with ring forceps and gently position the embryo as close to the uterine wall as possible.

(B) Higher-magnification images of an embryo from the boxed region in (A). The overlaid trace in the right image shows the location of the embryonic eye and lens.

(C) Method of AAV injection. AAV with 0.1% fast green is injected using a glass micropipette inserted into the eye just outside the lens.

(D) A successful injection is indicated by the fast green dye staying within and uniformly filling the embryonic eye.

(E) Image shows an exposed C57BL/6J embryo positioned for injection. The pigmented eye is clearly visible.

(F) Images show uninjected (left panel) and CTB488 injected (right panel) C57BL/6J embryos viewed under goggles (FHS/EF-2G2; BLS LTD., Hungary). Co-injection of a fluorescent dye can be used to assess the success of viral injections into pigmented eyes.

18. Holding the glass micropipette with your hand, insert the tip of the micropipette at a ~45° angle from the line of sight into the eye just outside the lens (~0.1 mm away from the lens) (Figure 3C).
19. Inject up to 0.5 μL of viral solution at a rate of 0.05 μL/s into the vitreous using the syringe pump.

Note: In mice with non-pigmented eyes (e.g., CD1), a successful injection is indicated by the fast green dye staying inside the eye (Figures 3D and 4A). If the injection is unsuccessful, the dye will diffuse outside the eye and not be visible. If this occurs, move on to the next embryo. This pup will need to be later excluded from analysis as it would likely have viral transduction in cells in the brain/outside the retina. As unsuccessfully injected embryos cannot be removed during surgery and will have to be identified after the pups are born, we always co-inject an AAV encoding a fluorescent protein. After birth, goggles (e.g., FHS/EF-2G2; BLS LTD., Hungary) can be used to identify and exclude unsuccessfully injected animals.

Note: For mice with pigmented eyes (e.g., C57BL/6), instead of fast green, fluorescent dyes in combination with goggles (e.g., FHS/EF-2G2) can be used to confirm successful injections (Figures 3E and 3F).

20. Repeat steps 13–19 with the other embryos.

⚠ CRITICAL: Longer procedure (>30 minutes from the exposure of embryos) decreases the survival rate. If the procedure time has reached 30 minutes, stop the injections to additional embryos and proceed to step 21.

Suturing and post-operative care

21. Return the uterus into the abdominal cavity and fill the abdominal cavity with warm saline.
22. Suture to close the abdominal wall and then the skin.

Alternatives: The skin layer may be closed with wound clips.

23. Place the mouse back in its home cage that is on a heating source (37°C, recirculating water heating pad) until the mouse recovers.
24. Inspect the mouse every day and provide analgesia for three days after surgery. The mouse should be inspected for any abnormalities to the surgical lesion (including wound dehiscence, swelling, discharge, rubbing or scratching of the surgical site) and for animal behavior (including appetite, grooming, and movement).

Note: In Yasuda et al. (2021), 5–10 mg/kg of Meloxicam was administered subcutaneously every 24 hours for three days as an analgesic. Other analgesics may be used, and the required dose should be administered as approved by the respective institutional animal care and use committees.

25. If fluorescent protein-expressing AAV was injected, at postnatal day 0 (P0), successful viral transduction can be determined using goggles (e.g., FHS/EF-2G2) (Figure 4B).

EXPECTED OUTCOMES

With AAV-DJ-CAG-EGFP, efficient viral transduction in the retina can be detected at P0. EGFP is highly expressed in the injected eye at P0 (Figure 4B). EGFP is uniformly expressed in the retina by P3, and the uniform expression persists at P15 (Figure 4C). The cross section of the infected retina (Figure 4D) shows that EGFP is highly expressed in the ganglion cell layer (GCL), where retinal ganglion cells (RGCs) and displaced amacrine cells are located ($72.9 \pm 4.0\%$ of cells in the GCL are EGFP positive). In the inner nuclear layer (INL), EGFP is highly expressed in horizontal cells and sparsely in amacrine cells and bipolar
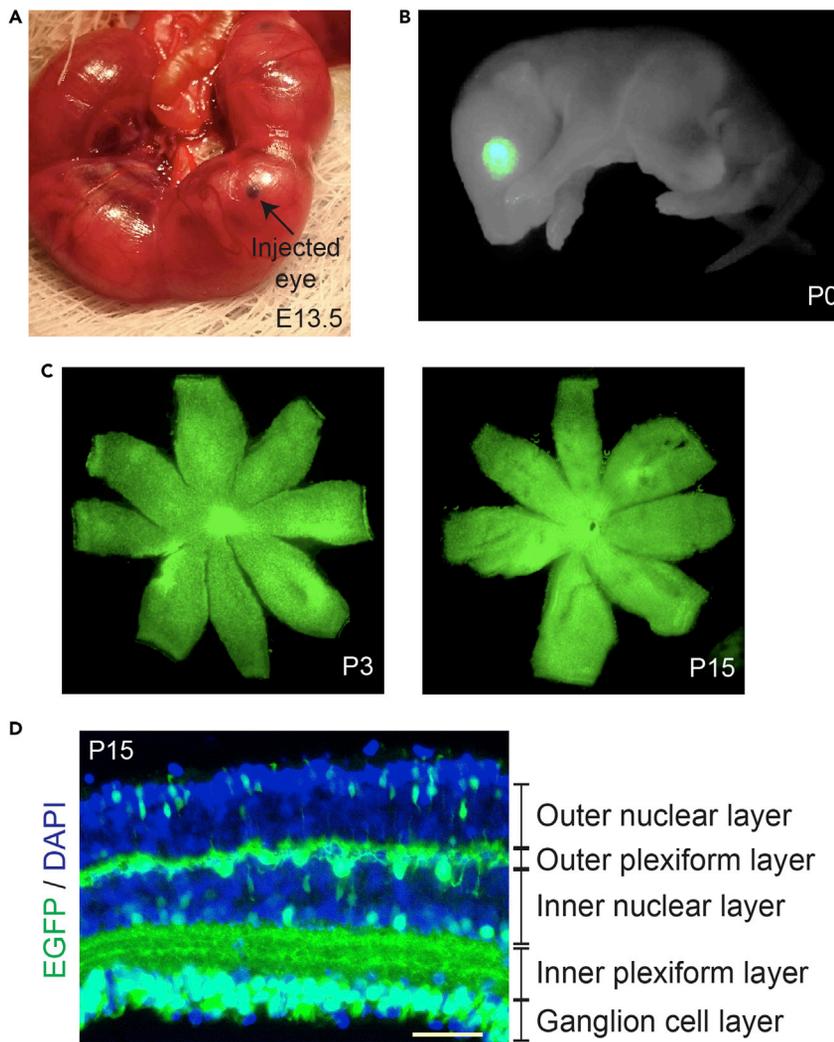
Figure 4. Efficient viral transduction in the early postnatal retina following *in utero* intraocular viral injections
(A) Embryo injected with AAV-DJ-CAG-EGFP (with 0.1% fast green dye) at E13.5.
(B) Expression of EGFP in a P0 pup eye taken with a goggle (FHS/EF-2G2; BLS LTD., Hungary) and Sony Xperia camera.
(C) Whole mount images of the injected retina. EGFP is highly and uniformly expressed in the retina at P3 and P15.
(D) Cross section image of the injected retina at P15. *In utero* intraocular injections of AAV-DJ-CAG-EGFP effectively labels RGCs and displaced amacrine cells in the GCL. Horizontal cells, amacrine cells and bipolar cells in the INL, and photoreceptors in the ONL are also infected. Scale bar, 50 μm.
Images in Figures 4B and 4C are from Yasuda et al., 2021.

cells. EGFP is also sparsely expressed in photoreceptors in the outer nuclear layer (ONL). Furthermore, EGFP-positive RGC axons are detected in the superior colliculus (data not shown) and dorsal lateral geniculate nuclei (dLGN; Figure 5). Thus, the time course of retinogeniculate pathway remodeling can be followed (Figure 5; Godement et al., 1984; Penn et al., 1998; Yasuda et al., 2021).

LIMITATIONS

In utero intraocular AAV injection is an effective method of manipulating gene expression in the retina. In this protocol, the AAV used (AAV-DJ) expresses the gene-of-interest under the CAG promoter. This resulted in a high level of gene expression in the cells in the GCL (Figure 4D). Cells in the INL and ONL are sparsely infected as well (Figure 4D). This expression pattern may be due to the AAV-DJ serotype used. AAVs of different serotypes are known to have different tropisms for different retinal cell types in the neonatal retina (Watanabe et al., 2013). Therefore, an appropriate
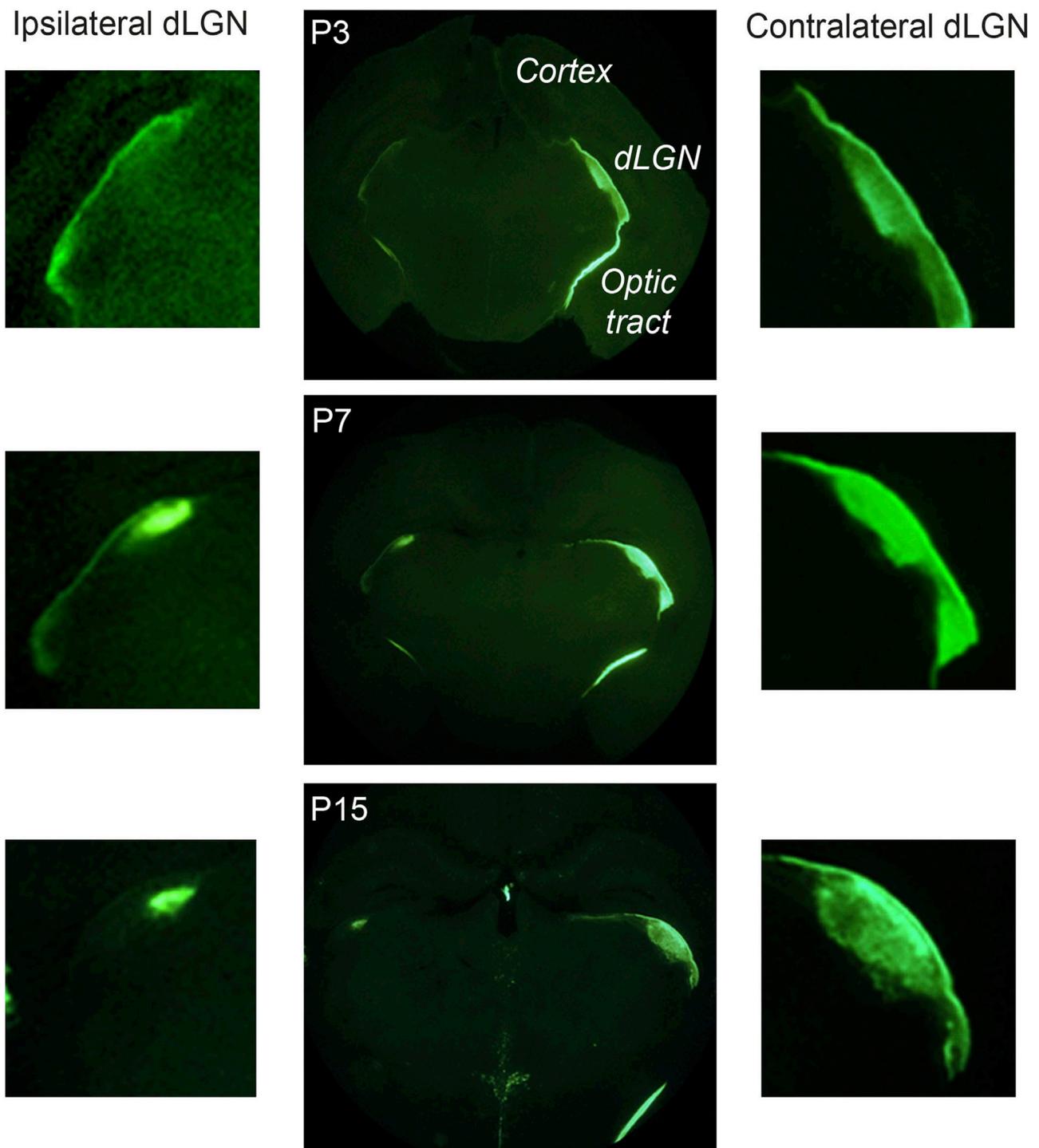
Ipsilateral dLGN

Contralateral dLGN

Figure 5. RGC axons are clearly detected in the developing dLGN following *in utero* intraocular viral injections

Ipsilateral and contralateral projecting EGFP-positive RGC axons can be detected along the optic tract and in the dLGN at P3, P7, and P15 following *in utero* intraocular AAV-DJ-CAG-EGFP injection into the left eye at E13.5.

AAV serotype should be used in each experiment. Additional cell-type specific gene expression can be achieved using cell-type specific promoters or Cre-dependent viruses in combination with cell-type specific Cre-lines. Loss-of-function of specific genes can be done via the introduction of

AAV-Cre into floxed animals. While this method facilitates broad manipulations of gene expression in the developing retina, there are a few limitations that should be considered.

Limitation 1: Number of embryos injected. The number of embryos that can be injected is limited by the length of surgery, the position of the embryo within the uterus, and the thickness of the uterine wall. Surgeries must be completed within 30 min (from the time of incision) to ensure high survival rates of the pregnant female and embryos. The number of embryos injected during this time can be further limited if the embryos are not ideally positioned within the uterus or if the uterine wall is too thick (the thickness of the uterine wall may vary between pregnant females) for clear identification of and injection into the eye. Handling and manipulation of the embryos should be minimized to improve survival. Nevertheless, with practice, > 80% of embryos (at least 10 embryos per pregnant CD1 mouse) can be injected.

Limitation 2: Viral transduction efficiency and consistency. Using this protocol, high and uniform gene expression in the retina can be obtained in > 90% of successfully injected (uniform fast green labeling of the eye) embryos. However, unsuccessfully injected embryos cannot be removed during surgery and will have to be identified after the pups are born. Therefore, we co-inject an AAV encoding a fluorescent protein to identify and exclude unsuccessfully injected animals. In all cases, evaluation of the efficiency and consistency of viral infections (e.g., via fluorescent protein expression or immunostaining) should be done for a proper interpretation of the data obtained.

TROUBLESHOOTING
Problem 1
Miscarriage of the pregnant female or low survival rates of the pregnant female and embryos post-surgery (related to steps 10–22).

Potential solution
Complete the surgery (beginning from the time of incision) within 30 min. Do not damage any blood vessels or the placenta. During the surgery, handle the embryos only by holding the uterine wall. Do not touch the placenta. Use ring forceps and not fine forceps or other sharp surgical tools to handle embryos.

Problem 2
Embryos are not ideally positioned within the uterine wall for intraocular injections (related to steps 14 and 15).

Potential solution
Gently turn the embryo within the uterus by pushing the embryo's back using ring forceps until the eye is facing up. Be careful to not damage or touch the placenta during this process. If positioning an embryo is too difficult, it is better to move on to the next embryo so that the procedure can be completed within 30 min from the time of incision.

Problem 3
Uneven or low viral transduction in the retina (related to step 19).

Potential solution
To verify successful AAV injections, add 0.1% fast green dye to the viral solution. In mice with non-pigmented eyes (e.g., CD1), an even and successful injection of virus is indicated by the fast green dye staying inside and evenly filling the eye (Figures 3D and 4A). If the dye appears diffuse or faint, the injection was unsuccessful and would result in uneven or low viral transduction. In mice with pigmented eyes (e.g., C57BL/6), this dye will not be visible and therefore cannot be used to indicate successful injections. In this case, fluorescent dyes can be used to monitor the successful injection of the virus solution into the embryonic eye (Figure 3F). Additionally, pigmented embryonic eyes are easier to identify during surgery, facilitating more successful viral injections.

Problem 4
Viral transduction is detected in cells in the brain/outside the retina (related to steps 18 and 19).

Potential solution
Cells outside the retina may be infected due to a leak if the micropipette is injected too deep or if the injection speed is too high. Ensure that the micropipette is inserted just into the eye, and reduce infusion speeds.

Problem 5
Injected pups have developmental abnormalities such as coloboma, microphthalmia, or the inability to open the eyelid (related to step 19).

Potential solution
To prevent these abnormalities, inject a small volume of AAV solution at a slow speed. The injected volume should not exceed 0.5 μL. Infusion speeds should also be reduced, and the narrowest possible micropipette (tip size < 50 μm) should be used.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Hisashi Umemori (hisashi.umemori@childrens.harvard.edu).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate/analyze new data sets or code.

AUTHOR CONTRIBUTIONS
Methodology, M.Y. and S.N.-C.; writing, M.Y., S.N.-C., and H.U.; supervision and funding acquisition, H.U.

DECLARATION OF INTERESTS
The authors declare no competing interest.

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