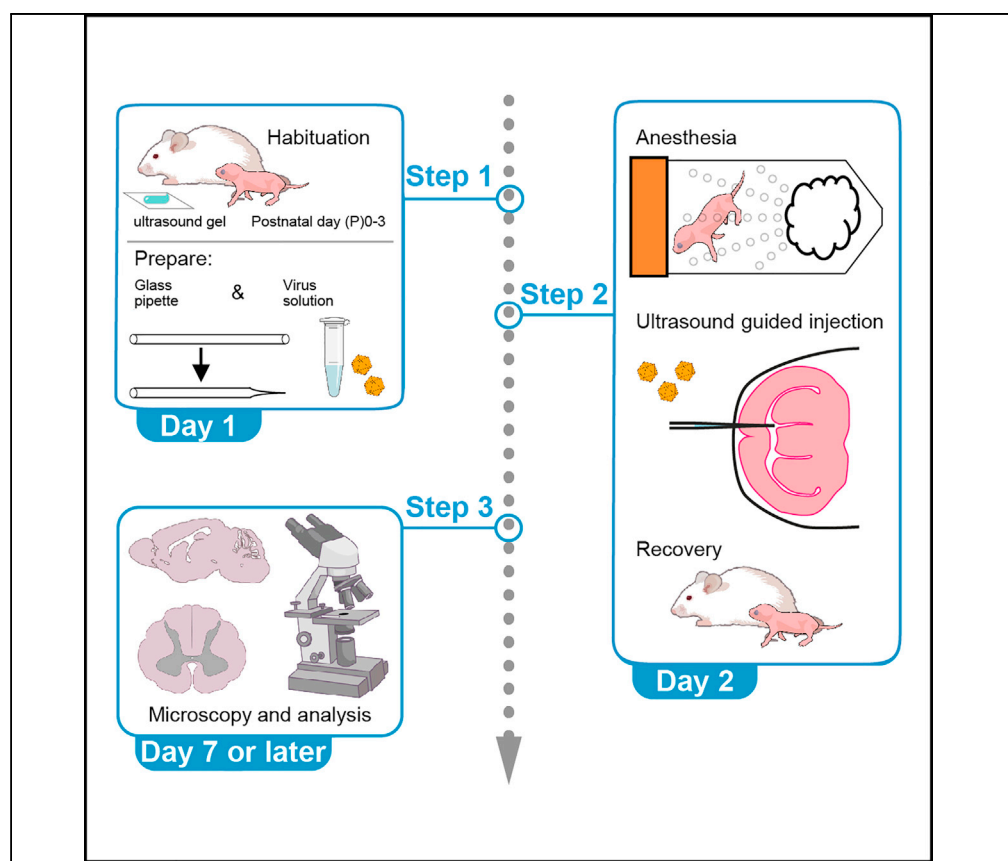


## Protocol

# Neural labeling and manipulation by neonatal intraventricular viral injection in mice



This step-by-step protocol provides a fast and easy technique to label and/or genetically manipulate neural cells, achieved by intraventricular injection of viral vectors into neonatal mice under ultrasound guidance. Successful injection of adeno-associated viral vectors (AAV) induces neural transduction as fast as 3 days post injection (dpi) in both the central and peripheral nervous systems. Virally driven expression persists until early adulthood. The same setup enables injection of other viral vectors as well as intramuscular injection.

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### Highlights

Protocol for  
intraventricular viral  
injection in neonatal  
mice under  
ultrasound guidance

Neural transduction  
as fast as 3 dpi and  
expression persists  
until adulthood

Fast and easy  
technique to label  
and/or genetically  
manipulate neural  
cells

Same setup can be  
adapted to inject  
intramuscularly or to  
inject other viral  
vectors

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## Protocol

# Neural labeling and manipulation by neonatal intraventricular viral injection in mice

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## SUMMARY

This step-by-step protocol provides a fast and easy technique to label and/or genetically manipulate neural cells, achieved by intraventricular injection of viral vectors into neonatal mice under ultrasound guidance. Successful injection of adeno-associated viral vectors (AAV) induces neural transduction as fast as 3 days post injection (dpi) in both the central and peripheral nervous systems. Virally driven expression persists until early adulthood. The same setup enables injection of other viral vectors as well as intramuscular injection.

For complete details on the use and execution of this protocol, please refer to Wang et al. (2021) and Brill et al. (2016).

## BEFORE YOU BEGIN

The below protocol describes injection of adeno-associated viral vectors (AAV) into the lateral ventricle of postnatal mice (day (P) 0–3), adapted from Kim et al. (2014) and Prendiville et al. (2014). We also applied this procedure to inject into muscles of postnatal animals (P 7). While we injected AAVs due to swift and neuron-biased expression, any other virus (e.g., recombinant lenti-, retro- or rabies viral vectors) or dyes could be utilized.

Cell-type specific genetic modifications or reporter-driven labeling are achieved by injection of AAV vectors encoding for Cre-recombinase, potentially carrying a cell-type specific promoter, into conditional knock-out mouse lines. Different AAV serotypes can lead to variable expression patterns and efficiency. In Brill et al. (2016) and Wang et al., (2021) we used AAV9 for motor axon labeling. Here we show examples using the PHP.eB capsid (Chan et al., 2017) to target predominantly neurons of the nervous system. Note that local regulations for biosafety and animal welfare can vary substantially and need to be considered first, requiring tailored modifications of this protocol. Here all animal experiments were approved by and performed in accordance with the regulations by the government of Upper Bavaria.

## Acquire mouse lines and AAV vectors of choice

⌚ Timing: >3 weeks

1. Obtain and cross stock mouse lines of the preferred genotype.



## 2. Acquire AAV vectors.

Critical factors are serotype, titer and length of expression cassette.

- Choose AAV serotype depending on desired expression pattern, for example, a neuron-biased expression pattern is induced by PHP.eB (see [Figure 3](#)).
- Ensure optimal titer of the used virus batch. We recommend  $> 10^{13}$  vector genomes (vg)/mL for AAV1/2 or AAV9, while  $> 10^{12}$  vg/mL for PHP.eB is sufficient.
- Injection of fewer vector genomes can lead to subset or single-cell labeling.
- The AAV vector cassette has a length limitation; for single stranded AAV  $\sim 4.5$  kb, or for higher transduction efficiency with double stranded AAV  $\sim 2.2$  kb ([McCarty et al., 2001](#); [Wang et al., 2003](#)).
- Store virus aliquots at  $-80^{\circ}\text{C}$ .

**△ CRITICAL:** We mostly use homozygous reporter lines in combination with a Cre-encoding AAV for inducing brighter fluorescent protein expression.

**Optional:** Consider plug check for timing the experiment.

Reporter lines (e.g., Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J, Jackson #7914; [Madisen et al., 2010](#)) allow tracing of targeted cells upon injection of Cre-encoding viral vectors without cassette for fluorescent protein expression (AAV-promoter-Cre) and provide a wide range of 'colors' and combinations.

If the experiment requires littermate controls, we suggest crossing heterozygous mice (e.g., flox/wild-type  $\times$  flox/wild-type or knock-out/wild-type  $\times$  knock-out/wild-type).

## Habituate mother with litter

⌚ Timing: 1 day

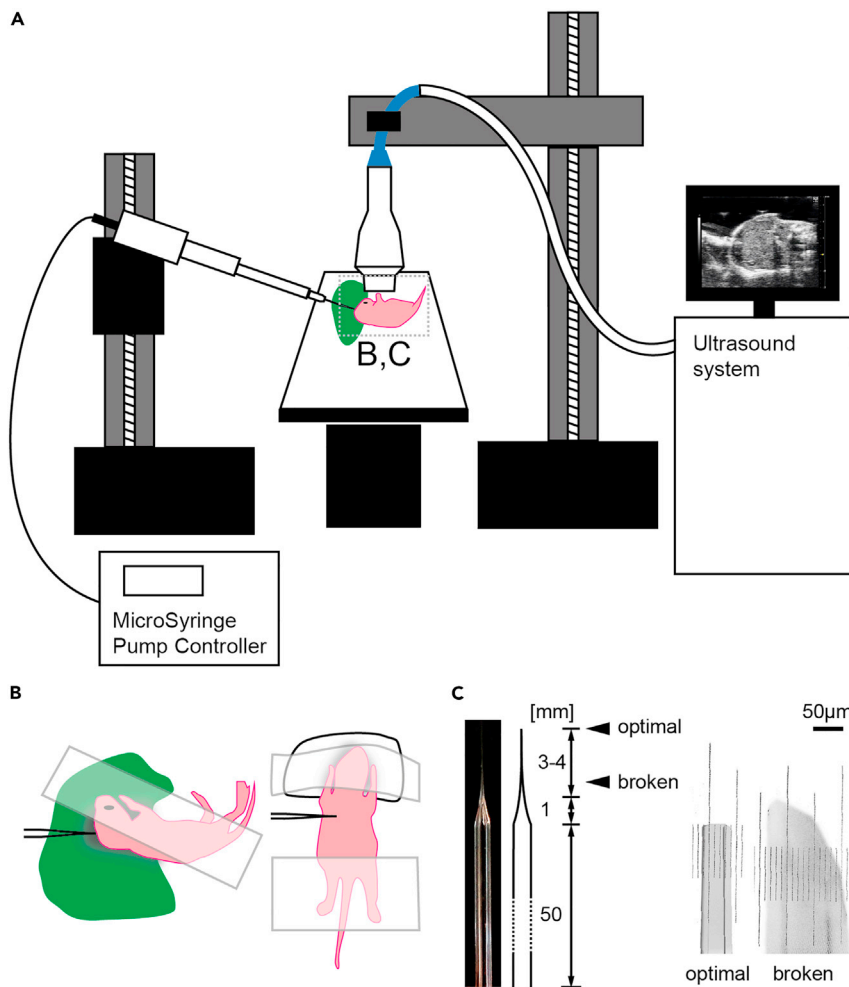
- Transfer female mouse together with newborn litter ([Figure 1](#), step 1 habituation on P0–3) to the experimental facility on the day before the actual injection procedure.
- Place a tissue paper with a small amount of ultrasound gel ( $\sim 1$  mL) inside the home cage for the female mouse to familiarize itself with the smell.

**△ CRITICAL:** Age of the pups at injection (P0–4) affects mortality and expression pattern. While neonatal P0–1 pups tend to have a higher mortality rate, systemic transduction is achieved more easily. As a compromise, we perform most of our injection experiments at P2.

## Prepare viral vector solution

⌚ Timing: 10 min

- Calculate the amount of virus needed for the experimental batch. We inject per pup  $> 3 \times 10^{10}$  vector genomes (vg) for AAV1/2 or AAV9, while  $> 7.5 \times 10^9$  vg for PHP.eB is sufficient. The maximum injection volume depends on the injection site, e.g., 5  $\mu\text{L}$  intramuscular (we usually inject 1  $\mu\text{L}$ ); 3  $\mu\text{L}$  intraventricular.
- Thaw appropriate amount of viral vector aliquots on ice. In order to maximize viral transduction efficiency, avoid freeze/thaw cycle by defrosting whole aliquots of the viral stock solution, and discard the leftover.
- Dilute viral solution with lactate-ringer solution to adjust titer according to virus activity and experimental aim.



**Figure 1. Setup for ultrasound guided neonatal injection**

(A) Schematic of injection setup with pump controller and ultrasound system.  
(B) Mouse pup position for injection: left, intraventricular injection; right: intramuscular injection.  
(C) Photo and schematic of pulled and cut needle.

8. Add 0.05 % (wt/vol) trypan blue to the viral injection solution for visualizing the correct filling of the injected ventricles. Centrifuge trypan blue and take supernatant to avoid crystals clogging the microinjection needle.

△ **CRITICAL:** Low viral titer could result in sparse and/or insufficient expression, while very high titers of virus might induce cytotoxicity.

If trypan blue precipitated, centrifuge solution. Add the clear supernatant only.

### Fill and mount microinjection needles

⌚ **Timing:** 10 min

9. Pull glass capillary at micropipette puller to create a long and thin needle tip (shape and size of needle see [Figure 1C](#)). Specific settings may vary. We use 3.5" Drummond glass pipettes, 3-000-203-G/X, with 0.53 mm inner diameter, 1.14mm outer diameter, and 88.9 mm length (without

- filament); settings: Heat 530; Pull 230; Velocity 70; Delay 40; Pressure 500; Ramp 536; Flaming/Brown puller (Model P-1000, Sutter Instrument with box heating filament).
10. Cut the tip of the glass micropipette with forceps to create an opening of  $\sim 30 \mu\text{m}$  diameter (Figure 1C).
  11. Fill the glass micropipette from the back with mineral oil with an insulin syringe or a 30 G needle and 1 mL syringe.
  12. Mount filled glass micropipette on the injection device by passing the metal plunger of the injector through the micropipette, and the thicker end of the micropipette should be fixed in the rubber ring of the injector.
  13. Withdraw the volume of viral particles required before anesthetizing the pup (1–3  $\mu\text{L}$ ).

**△ CRITICAL:** The cut of the micropipette should be made where the pipette tip stops being bendy, to create a sharp but sturdy tip (Figure 1C). When backfilling, the mineral oil should go in with ease. If not, cut the tip more to create a larger opening.

A fresh glass pipette must be used for each viral vector, while the same pipette can be refilled multiple times if injecting the same viral vector and if the tip of the pipette remains sharp.

**Optional:** Finer tips for microinjection needles could be created with a microelectrode beveller.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Bacterial and virus strains</b>		
AAV PHP.eB- CAG-tdTomato	Addgene	59462-PHPeB
<b>Chemicals, peptides, and recombinant proteins</b>		
Sodium dihydrogen phosphate dihydrate ( $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ , for 0.1M PB)	Carl Roth	Cat# 2370.2
Disodium hydrogen phosphate dihydrate ( $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , for 0.1M PB)	Carl Roth	Cat# 4984.1
Vectashield antifade mounting medium	Vector Laboratories	Cat# H-1000
Trypan blue, 0.4% solution	Lonza	Cat# 17-942E
Ringer-lactate solution	B. Braun	Cat# 9515677
<b>Experimental models: Organisms/strains</b>		
C57BL/6J	The Jackson Laboratory	JAX: 000664 IMSR_JAX:000664
<b>Software and algorithms</b>		
Fiji ImageJ	Schindelin et al., 2012	<a href="http://fiji.sc">http://fiji.sc</a> RRID:SCR_002285
Adobe Photoshop CS6	Adobe	<a href="https://www.adobe.com">https://www.adobe.com</a> RRID:SCR_014199
Adobe Illustrator	Adobe	<a href="https://www.adobe.com">https://www.adobe.com</a> RRID:SCR_010279
<b>Other</b>		
fine glass pipette (3.5")	Drummond	Cat# 3-000-203-G/X
Flaming/Brown puller (Model P-1000)	Sutter Instrument	Cat# P-1000
Micro syringe pump controller	World Precision Instruments	Micro4
Nanoliter injector	World Precision Instruments	Nanoliter 2000 WPI #B203XV
Ultrasound imaging system	Visualsonics	Vevo 1100
Microscan transducer	Visualsonics	MS550D
3M™ Micropore™ Surgical Tape	3M	F51DA01
Animal tattoo ink paste	Ketchum	Cat# 329A

## STEP-BY-STEP METHOD DETAILS

### Anesthesia of neonatal mouse

⌚ Timing: up to 3 min; ~30 s anesthesia

This step describes the anesthesia of neonatal mice before injection. The pups are anesthetized separately right before injection with a high concentration of isoflurane. Lower concentration of isoflurane or injection anesthesia are less efficient and lead to higher mortality rate. Alternatively – where permitted by the local authorities – hypothermia could serve for anesthetizing neonates.

1. Separate the whole litter of pups from the mother and place onto a heated pad (~35°C) next to the injection setup. Add bedding and nesting material to ensure comfort and to enable pups to retain maternal smell. All injected pups are returned together to the home cage following recovery.
2. Add 0.5 mL isoflurane to a 50 mL falcon tube padded with tissue paper and close the lid tightly, in order to quickly achieve a high concentration of vaporized isoflurane.
3. Place one neonate inside the falcon tube and close the lid. Observe closely for the moment the pup stops struggling and immediately take it out. Test anesthesia by light toe pinching. If you get a reaction, put it back for an additional 10 s of anesthesia.

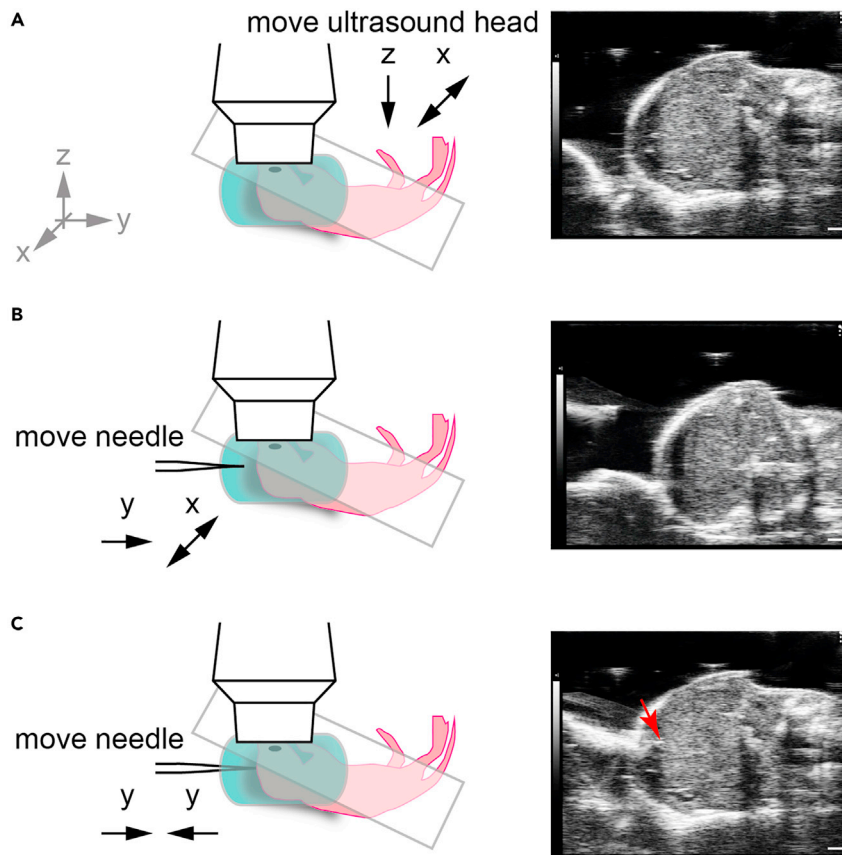
⚠ **CRITICAL:** To minimize lethality, the anesthetizing time should be kept as short as possible. Take care that pups do not come into direct contact with liquid isoflurane to avoid skin irritation. For very young mice (P0–2), the pup could be taken out shortly before all movement stops, since the remaining anesthetic in the airway is enough to induce anesthesia for the injection.

### Injection of viral vectors

⌚ Timing: up to 5 min

This step describes the injection of viral vectors (for example AAV9, PHP.eB) into the lateral ventricle of neonatal mice under ultrasound guidance (we use the Ultrasound imaging system Vevo 1100 with a microscan transducer MS550D (22–55 MHz, resolution ~50 µm, imaging depth 12 mm), Visualsonics. Alternatively, any ultrasound system providing a similar resolution and imaging depth should suffice). If high expression levels are required and available viral titer is low, several injections of neonates on consequent days are a possible solution (for example injection on P2 and P3). If different viruses are injected into the same litter, marking the pups (e.g., by tattooing on the paws) is essential.

4. Fix pup on the injection stage with tape (ideally a gentle-adhesive tape leaving no glue residue on the skin), cover pup's head with ample ultrasound gel ([Figure 1B](#)).
5. Pre-positioning of microinjection needle for actual ventricular injection.
  - a. Find lateral ventricle for injection ([Figure 2A](#); [Methods Video S1](#)).
    - i. Dip ultrasound head into ultrasound gel covering the mouse pup, lower (in z-direction, [Figure 2A](#)) until live images of brain structures appear on the ultrasound screen.
    - ii. Move ultrasound head laterally (in x-direction, [Figure 2A](#)) to find the injection plane, where the ventricle appears largest.
  - b. Align head of ultrasound device with previously mounted injection needle ([Figure 2B](#)).
    - i. The microinjection needle is set to be parallel to the ultrasound head. Adjust the needle to approach the head (in y-direction, [Figure 2B](#)) until the tip of the micropipette is dipped in ultrasound gel, under the ultrasound head and in vicinity of the skull.

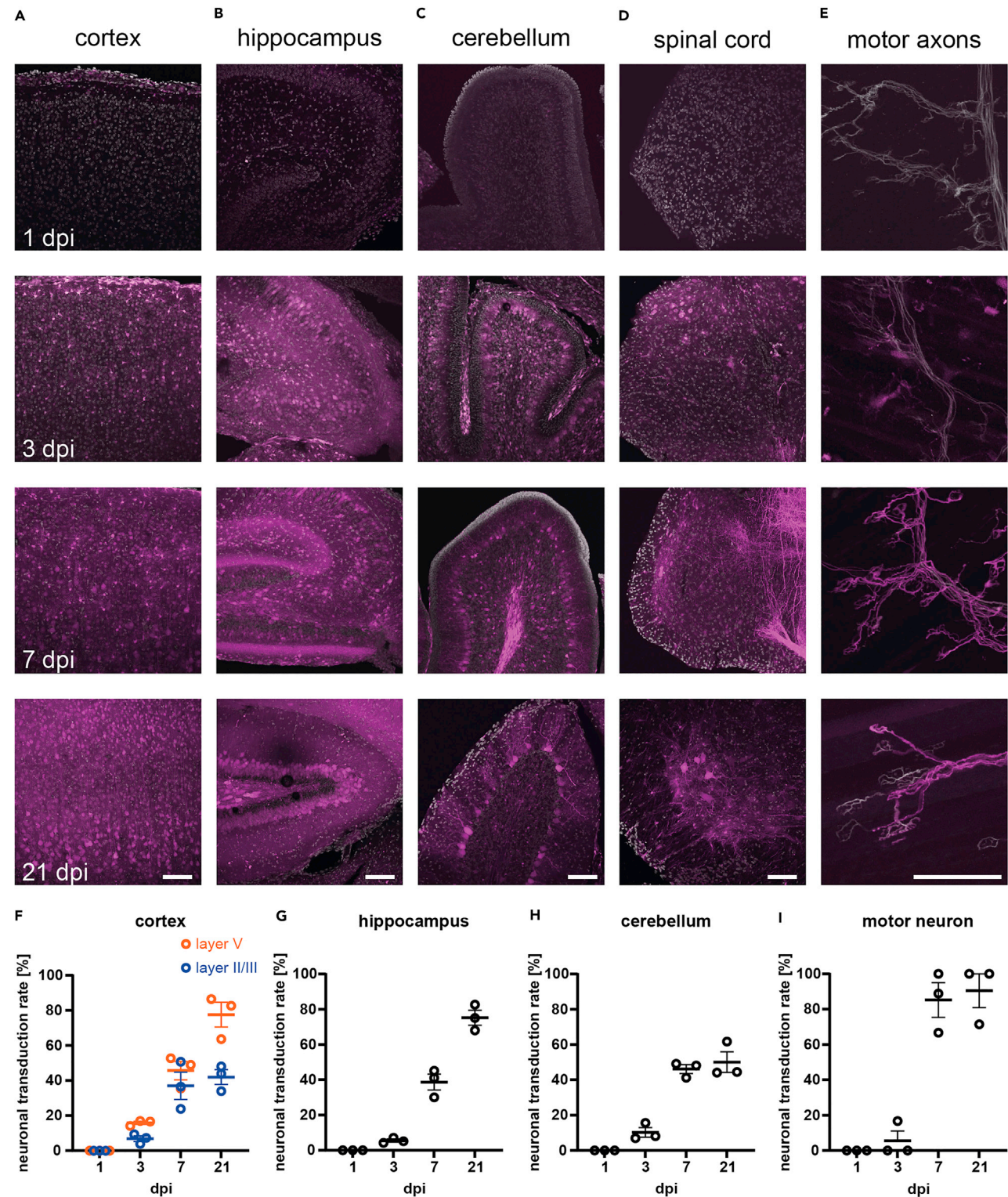


**Figure 2. Alignment and injection process**

(A–C) Schematic and sonograms of (A) moving down the ultrasound head and adjust to find the plane of lateral ventricles (shape marked in pink; directions shown in xyz coordinates); (B) aligning needle parallel to ultrasound head, then moving the needle tip to the vicinity of the pup's head and adjusting in x-direction to appear in the ultrasound imaging plane; and (C) inserting the needle tip into the right lateral ventricle, with needle tip visible on the right image (indicated by red arrow; also see [Methods Video S1](#)), then retracting after injecting the required amount of viral solution. Scale bars, 1mm.

- ii. Move the microinjection needle (in x-direction, [Figure 2B](#)) until the needle is aligned with the correct ultrasound plane and becomes focused on the ultrasound screen.
6. Injection of viral vectors ([Figure 2C](#); [Methods Video S1](#)).
  - a. Penetrate the pup's skull until the tip of the microinjection needle is inside the lateral ventricle. If the skull dents during this step (a small dent is common (see [Methods Video S1](#)) and does not affect skull integrity or transduction. If the dent is significantly > 0.5 mm, abort injection and exchange the needle), retract the micropipette once the tip reaches the designated spot until the skull regains its proper shape.
  - b. Inject up to 3  $\mu$ L of viral solution into the lateral ventricle at a flux rate of 30 nL/s.
  - c. Withdraw injection needle. When the pup is very young (P1 or 2), and/or using albino mouse-lines, the trypan-blue can be seen filling up the ventricle if injected correctly.
7. Recovery of injected mice. Gently remove remaining ultrasound gel from pup with soft tissue and return pup on heating pad ( $\sim 35^{\circ}\text{C}$ ). Return pups to the mother only when fully awake (breathing normally and can adjust body posture autonomously) and skin shows healthy pink coloring.
8. Virally driven expression of fluorescent proteins is detected as early as 3 days post injection, achieving broad peripheral and central nervous system transduction and persisting until early adulthood ([Figure 3](#)). We even experienced continued expression up to 1 year post injection.





**Figure 3. Expression of PHP.eB-CAG-TdTomato in C57BL/6J mouse**  
(A–I) Expression of TdTomato (magenta) in (A) cortex, (B) hippocampus, (C) cerebellum and (D) spinal cord ventral horn, counter stained with DAPI (white); and (E) motor axons innervating triangularis sterni muscle, counter stained with tubulin-βIII (white); (F–I) Quantification of the percentage of



**Figure 3. Continued**

TdTomato positive cells of NeuroTrace stained neurons in (F) Layer II/III (orange) and V (blue) motor cortex, (G) hippocampus CA1 region, (H) cerebellum and (I) *triangularis sterni* motor neurons (N=3 animals per time point, >50 neurons/axonal branches per animal analyzed). Data are represented as mean  $\pm$  SEM. Scale bars, 100  $\mu$ m, scale bars in 21 dpi applies to 1/3/7 dpi in the respective regions.

**⚠ CRITICAL:** To minimize lethality, the injection should be performed as fast as possible. We usually keep the injection process under 5 minutes per mouse pup. However, the injection flux rate should not exceed 30 nL/s (see step 6), since otherwise it could result in increased intracranial pressure.

**Note:** Skin bleeding is usually a sign of incorrect injection depth and/or blunt needle tip. Replace the microinjection needle in this case.

**Optional:** Intramuscular injection of small volume ( $\sim$ 1  $\mu$ L) of viral vectors is possible with this method, and no ultrasound guidance is needed here. For details see [Wang et al. \(2021\)](#).

**Optional:** Mark mouse pups by tattooing on paws.

**Optional:** If genotyping is required to identify experimental animals, obtain tail biopsies.

**Optional:** Offer oral analgesia such as Meloxicam ( $\sim$ 5  $\mu$ g per pup, e.g. 10  $\mu$ L of 0.5  $\mu$ g/ $\mu$ L solution).

**⏸ Pause Point:** In general, we highly recommend finishing the whole injection procedure rapidly. Short pauses can be taken between injections of each pup. For breaks longer than 10 min, return the litter to the mother/home cage.

## EXPECTED OUTCOMES

AAV vectors have become a widely used tool for gene delivery. This delivery method provides outstanding safety and efficiency, as well as applications for gene therapy. Compared to systemic delivery through intravenous injection in adult mice, the neonatal intraventricular injection achieves rapid expression in both the peripheral and central nervous systems as early as 3 dpi ([Figure 3](#)). Therefore, this technique not only provides a powerful approach for studying molecular mechanisms during postnatal development, but at the same time allows studies on adult mice as expression persists. In general, prolonged expression after injection results in brighter and more widespread labeling of transduced cells. In our experience, the brightness increases at least until 3 weeks post injection ([Figures 3F–3I](#)). Glia might appear overall brighter compared to big neurons due to their limited cellular volume. Further factors mediating brightness are a combination of mouse genotype (e.g., strength of reporter promoter, homo- or heterozygous reporter gene etc.) and vector design, as well as the capsid serotype ([Figure 3](#) demonstrates strong neuronal expression with the PHP.eB capsid). For genetic modifications, viruses encoding the gene of interest or a dominant negative version can be utilized (examples see [Wang et al., 2021](#)). In addition, more complex gene-manipulating systems are possible, such as Cre-recombinase encoding viruses injected into transgenic mice with floxed alleles to create conditional knock-out animals (examples see [Brill et al., 2016](#); [Wang et al., 2021](#)). Cell type, as well as subcellular organelle specificity can be accomplished through respective promoters (e.g., AAV-hSynapsin-Cre drives Cre-recombinase expression in neurons) and/or organelle tags (e.g., mitochondrial or endoplasmic reticulum targeting sequences drive expression in the respective organelles).

In this protocol, we analyzed an outcome from injecting PHP.eB-CAG-TdTomato ([Chan et al., 2017](#)), a capsid mainly targeting neuronal cells, into C57BL/6J mice. We demonstrate transduction of motor axons and neurons in various brain regions such as cortex, hippocampus and cerebellum, as early as 3 dpi. The percentage of transfected cells as well as expression strength increases with time, and

reaches maximum around 3 weeks post injection (Figure 3). For examples of genetic modification via Cre-driven conditional knock out and/or with dominant negative expression, please refer to Brill et al., (2016) and Wang et al., (2021).

## LIMITATIONS

The success of the transduction is largely dependent on the efficacy of the viral vectors. Besides the quality (purity and potency, largely depends on plasmid quality and manufacturer) of AAV virus, we recommend injection of minimum amount of  $3 \times 10^{10}$  vg/mouse for most serotypes (AAV1/2, AAV9). As we have very occasionally observed inflammation around the injected area (i.e., enlarged ventricles and/or apoptotic cells around the injection site) with a high titer of AAV (e.g.,  $7.5 \times 10^{10}$  vg/mouse of PHP.eB-CAG-TdTomato) 3 weeks post injection, balancing expression strength and cytotoxicity is a critical experimental factor.

Variability of expression could also depend on proper viral injection into the target area, such as ventricles or muscles. Selection of the correct injection location based on ultrasound image may be the most critical factor for the success of the experiment. An initial practice round of 5–10 pups is usually sufficient for training, where P3 or 4 animals are recommended for reduced lethality.

We observed some mouse lines are more susceptible to isoflurane anesthesia, where the anesthesia amount and time should be adjusted accordingly to reduce lethality. Aggression of the female mouse can result in rejection of weakened pups after injection. However, in general, the lethality rate is very low for experienced personnel, and over 95% of the pups should survive the procedure on a regular basis.

## TROUBLESHOOTING

### Problem 1

The capillary tip did not hit the ventricle during viral injection (refer to steps 5 and 6)

#### Potential solution

Align ultrasound head with lateral ventricle according to description in step 5. Since the tip of the microinjection needle can bend out of the ultrasound imaging plane, slight adjustment of the ultrasound head can help locate the exact position of the needle tip.

### Problem 2

Capillary gets clogged and/or stuck during injection (refer to step 6).

#### Potential solution

Abort injection and let pup fully recover from anesthesia before injecting again.

Decide:

If trypan blue dye crystallized, switch to a fresh capillary for the next animal. Centrifuge the dye every time before use.

If the opening of the micropipette tip is insufficient, cut the needle tip again.

### Problem 3

Bleeding at the injection site after withdrawing the injection needle (refer to step 6c).

#### Potential solution

Abort injection, disinfect the bleeding site and observe the pup closely for at least one day before injecting the same pup again. If the pup shows signs of distress that qualifies for discontinuation of the experiment, euthanize the pup.

Change for a fresh and optimally sharp needle before injecting the next pup.

**Problem 4**

Death of mouse pups after injection (refer to step 7)

**Potential solution**

For pups having breathing difficulties during the recovery phase, gentle and rapid (2–3 times/s) chest pressing helps to resume spontaneous breathing. We experienced a dramatic survival increase with this method.

If breathing difficulties are due to anesthetic depth, reduce time of isoflurane exposure (see step 3).

Return pups to mother only after the pups fully recover, i.e., breathing normally, can autonomously readjust body posture and showing healthy pink coloring.

To reduce the risk of mother rejecting pups after injection, keep some bedding with the pups during recovery. Always remove and return whole litters.

**Problem 5**

Transduction rate is lower than expected (refer to step 8).

**Potential solution**

Use viruses of higher quality (purity and/or potency) or from a different batch and/or manufacturer.

Increase viral titer and/or inject on an earlier postnatal day.

Inject the same pups twice, e.g., on P2 and P3.

Switch to a capsid with a higher expression rate.

**RESOURCE AVAILABILITY****Lead contact**

Further information and requests for resources and reagents should be directed to the lead contact, Monika Brill ([Monika.Leischner-Brill@tum.de](mailto:Monika.Leischner-Brill@tum.de)).

**Materials availability**

This study did not generate new unique reagents.

**Data and code availability**

The datasets supporting the current study have not been deposited in a public repository, but are available from the corresponding author on request.

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2021.101081>.

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## AUTHOR CONTRIBUTIONS

Conceptualization, M.W. and M.S.B.; investigation, M.W.; writing – original draft, M.W. and M.S.B.; writing – review & editing, all authors; supervision, M.S.B.; funding acquisition, M.S.B. and T.M.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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