Stereotaxic gene delivery in the rodent brain

Ali Cetin¹, Shoji Komai², Marina Eliava³, Peter H Seeburg¹ & Pavel Osten³

¹Department of Molecular Neurobiology, Max Planck Institute for Medical Research, Jahnstrasse 29, 69120 Heidelberg, Germany. ²Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0192, Japan. ³Department of Physiology, Feinberg School of Medicine, Northwestern University, 303 East Chicago Avenue, Chicago, Illinois 60611, USA. Correspondence should be addressed to P.O. (p-osten@northwestern.edu).

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Stereotaxic surgery has been an invaluable tool in systems neuroscience, applied in many experiments for the creation of sitetargeted lesions, injection of anatomical tracers or implantation of electrodes or microdialysis probes. In this protocol, we describe stereotaxic surgery optimized for gene delivery by recombinant adeno-associated viruses and lentiviruses in mice and rats. This method allows the manipulation of gene expression in the rodent brain with excellent spatiotemporal control; essentially any brain region of choice can be targeted and cells (or a subpopulation of cells) in that region can be stably genetically altered at any postnatal developmental stage up to adulthood. Many aspects of the method, its versatility, ease of application and high reproducibility, make it an attractive approach for studying genetic, cellular and circuit functions in the brain. The entire protocol can be completed in 1–2 hours.

INTRODUCTION

This protocol describes stereotaxic surgery for the delivery of recombinant adeno-associated viruses (rAAVs) and lentiviruses in the mouse and rat. The surgery is optimized to result in minimal injury to the brain parenchyma. The procedure is easy to adapt and provides reproducible results in terms of both the stereotaxic targeting and the volume of the injected virus. Many laboratories, including ours, have demonstrated that rAAVs and lentiviruses stably infect neurons *in vivo* without any associated toxicity^{1–3}. Hence, this protocol can be used to introduce stable genetic manipulations (heterologous gene expression or 'knockdown' of endogenous gene(s) by expression of short interfering RNA) in specific brain regions, essentially at any given stage of postnatal life.

The stereotactic gene delivery does not replace standard mouse genetics, but provides an alternative tool for researchers who wish to study the cellular mechanisms of brain function *in vivo*. Below are listed several considerations that may provide help in choosing the right approach for specific experimental manipulations.

Principles of stereotaxic targeting

Stereotaxic coordinates for any brain region can be easily determined from stereotaxic atlases such as *The Mouse Brain in* Stereotaxic Coordinates (George Paxinos and Keith B.J. Franklin, Academic Press, 2005) and *The Rat Brain in Stereotaxic Coordinates* (George Paxinos and Charles Watson, Academic Press, 2004). The coordinates are given as three-dimensional (x, y and z) distances (in mm) from bregma, which is the intersection of the coronal and sagittal sutures on the surface of the skull: the x plane represents the medial-to-lateral (left-to-right) distance from bregma, the y plane represents the rostral-to-caudal (front-to-back) distance from bregma and the z plane represents the dorsal-to-ventral (up-and-down) distance from bregma (**Box 1, Table 1**).

At what age can animals be used for stereotaxic surgery?

The mixture of ketamine and xylazine anesthesia described in this protocol can be used for work with very young animals, approximately from postnatal day 7 (P7), to adult animals (note that a lower dose and atropine before anesthesia is used for young animals; discussed further below). As the high metabolic rates of mice and rats (heart rates, 300–650 and 250–370 per min, and respiratory rates, 150–200 and 70–115 per min, for mouse and rat, respectively) limit the duration of injected anesthesia, it is very important to monitor closely, at 10-minute intervals, the

BOX 1 STEREOTAXIC TARGETING

Bone sutures on a skull surface determine the two landmarks used for stereotaxic targeting of specific brain regions: the bregma point is formed by the cross of the coronal and sagittal sutures, whereas the lambda point is the cross of the sagittal and lambdoid sutures (**Fig. 4**). For horizontal leveling of the animal's head (done by adjustment of the dorsal-ventral position of the nose clamp), both bregma and lambda are set at the same dorsoventral level; this is done by lowering a micropipette in the stereotaxic holder so it touches either lambda or bregma and comparing the z coordinates for the two points.

Stereotaxic coordinates for specific brain regions, such as those in **Table 1**, are easily determined from a brain atlas (e.g., *The Mouse Brain in Stereotaxic Coordinates* by Paxinos and Franklin) that lists rostral-caudal, medial-lateral and dorsal-ventral distances from bregma, which is assigned position 0. To obtain targeting coordinates for a specific injection region, subtract the atlas coordinates from the position of the animal's bregma in the stereotaxic apparatus. For example, if the position of bregma (in mm) in the stereotaxic frame is caudal-rostral 61.5, medial-lateral 12.0 and dorsal-ventral 35.5, the target coordinates (in mm) for subthalamic nucleus will be caudal, 63.4; lateral, 13.6; and ventral, 31.1 (note that the frame of the Kopf stereotaxic apparatus used in our lab adds caudal and subtracts rostral distance from the position of bregma).

anesthesia depth throughout the surgery; easy parameters to assess are a relaxed respiratory rate and absent withdrawal reflex to foot pinch.

Volatile anesthesia with isoflurane is a convenient method for adult animals⁴. However, isoflurane has been shown to induce neurodegeneration in the brain of P7 rats after prolonged application (6 h)⁵, suggesting that it should be used with caution and only for brief periods in young animals. In addition, mice and rats younger than P7 may be anesthetized by deep hypothermia^{6,7}; however, we do not have experience with that procedure and thus it is not described in this protocol.

Brain regions of what size can be infected with a single injection site?

The size of the brain region that can be infected differs considerably for young versus adult animals, as well as for rAAV versus lentiviral vectors. Young animals have a larger extracellular space, which allows for less hindrance and thus better spread of recombinant viral particles in the brain parenchyma. The difference in the extracellular space spread for the two recombinant viruses (rAAVs spread much better than lentiviruses) is a result of the different diameters of the viral particles: 20-30 nm for rAAV particles and over 100 nm for lentiviral particles8. As the extracellular space width of the adult rodent brain is approximately 40-60 nm^{9,10}, the hindrance for the spread of lentiviruses is considerable. In our experience, it is difficult to obtain an infection volume with diameter larger than 500-700 µm from a single injection of lentivirus, at least in the cortex and hippocampus³. The rAAV particles spread much better in the rodent brain extracellular space, and that may be further enhanced by convection-enhanced delivery^{11,12}, co-injection of heparin¹³ or co-injection of mannitol¹⁴. With these protocols, many groups have described infection of fairly large brain regions, typically using injections of 1-2 µl of viral solution in the cortex, hippocampus and striatum¹⁵⁻¹⁷. However, when applying modifications to increase the volume of the infected region, it is important to keep in mind that the brain is a 'closed container' and that increasing intracranial pressure by delivering larger volumes can easily lead to irreversible damage of the brain parenchyma.

What cell-type specificity can be achieved with the rAAV and lentiviral systems?

Stereotaxic delivery allows targeting of a specific brain region. In such regions, however, there are usually different neuronal cell types, characterized by distinct synaptic, biophysical, biochemi-

 Table 1 | Examples of stereotaxic coordinates.

cal and/or morphological properties¹⁸. In principle, distinct neuronal cell types can be targeted by two approaches: the virus can be manipulated to infect only the target cells; and the viral vector can be constructed to drive expression only in the target cells. The first option is very limited for both rAAVs and lentiviruses, even though some cell-type selectivity has been described for rAAV of the 1, 2, 4 and 5 serotypes^{19,20}. Lentiviruses are typically produced (pseudotyped) with surface glycoproteins derived from vesicular stomatitis virus²¹, which gives them very broad host range²²; however, pseudotyping of equine infectious anemia virus, a lentivirus isolated from horses, with rabies glycoproteins leads to selective uptake of the lentivirus by nerve terminals²³. With this method, it is possible to stably label (and manipulate) neurons that project to the stereotaxically targeted area (P.O., M.E. and A.C, unpublished results). Both the rAAV capsid protein and lentiviral surface glycoprotein can be engineered to recognize cell type-specific surface receptors^{24,25}; however, such methods have not yet been applied for cell-type targeting of neuronal cells.

The second option for achieving cell type-specific expression is to construct a vector with recombinant promoter derived from a gene expressed only in the targeted cell type in the specific brain region. For example, neuron-specific expression has been achieved with lentivirus and rAAV vectors using promoters of the genes encoding synapsin¹⁶ and neuron-specific enolase¹⁵, and pyramidal neuron-specific expression has been achieved with promoters of the genes encoding Thy-1.2 and CaMKII (ref. 26). More-selective expression targeting specific neuronal subtypes, although highly desirable for many experimental applications¹⁸, is yet to be achieved.

What experimental applications are most suitable for stereotaxic gene delivery?

Because of the limited spread of viral particles in the brain parenchyma (described above), viral vector-based genetics are best suited for cellular studies that require infection of relatively small brain regions. For example, essentially any cell in the brain can be labeled using expression of green fluorescence protein (GFP) and can be functionally altered by coexpression of a heterologous gene or short interfering RNA to induce gene 'knockdown'. The consequences of such manipulations can be analyzed by electrophysiology in acutely prepared slices from infected animals²⁶; in this case, relatively sparse infection offers the possibility of assessing infected (experimental) cells and uninfected (control) cells next to each other in the same brain slice. Another well suited application is the use of recombinant viruses for anatomical tracing; for

Targeted region adult mouse brain)	Rostral (+) and caudal (–) (mm)	Lateral (mm)	Ventral (mm)
Subthalamic nucleus	- 1.9	1.6	4.4
Dorsal hippocampus, CA1	- 2.1	2.0	1.4
Basolateral amygdala	- 1.5	2.75	4.75
Lateral ventricle	+ 0.5	0.75	2.5
Nucleus accumbens, core	+ 1.1	1.2	4.5

example, simply labeling the entire axonal arborization with GFP²⁷. Finally, *in vivo* cellular analysis of virally infected and manipulated cortical cells is possible in anesthetized rats and mice with the two-photon targeted-patching technique^{28–30}.

As for the study of gene function in behavior, several groups have used viral vectors to infect specific brains regions in mice, such as the midbrain ventral tegmental area and lateral amygdala, and have demonstrated distinct behavioral phenotypes^{31,32}. The main advantage of this approach lies in the exceptional spatiotemporal control over the induced genetic manipulation, which avoids the confounding variables, such as gene alterations during early development and/or in multiple brain regions, typically associated with traditional mouse genetics.

MATERIALS

REAGENTS

- Ethanol, 70% (disinfectant (e.g., Meritz Plus; Medline Industries)
- Mice or rats (either species can be used for gene delivery via viral vectors with the same efficiency)
 CAUTION All experiments must be done in accordance with guidelines and regulations of the relevant authorities.
- Anesthetics and analgesics³³ (ketamine, xylazine, lidocaine, buprenorphine and meloxicam)
 CAUTION Ketamine and buprenorphine are narcotics and should be handled according to relevant rules of the host institution.
- Atropine (optional)
- Lubricant eye ointment
- Sterile PBS
- Purified rAAV or lentivirus
- Bone wax
- Triple antibiotic ointment

EQUIPMENT

- Surgical tools (e.g., Fine Science Tools), including small surgical scalpel and scissors, small dull forceps, fine sharp forceps (#5), a small bone scraper and 'surgical hooks' (made from clipped and bent needles)
- Laboratory scale
- Electric hair shaver
- Small animal stereotaxic apparatus (such as Kopf, model 963 or 940) with nonrupture ear bars (such as Kopf, model 922 for mouse and 955 for rat)
- Dissecting microscope (such as the Leica S6E; total magnification should be at least an objective of 40×-4× plus 10× eyepieces) and microscope boom stand (such as the SMS25 from Diagnostic Instruments)
- Cotton swabs
- Hand-held drill (such as the Osada EXL-M40) with small dental drill bits (such as the Brasseler H1.21.005)
- 5-µl calibrated micropipettes (VWR or Blaubrand)
- Micropipette puller (such as the Sutter P-97)
- Syringes (1, 5 and 20 ml) and needles (23 G and 27 G)

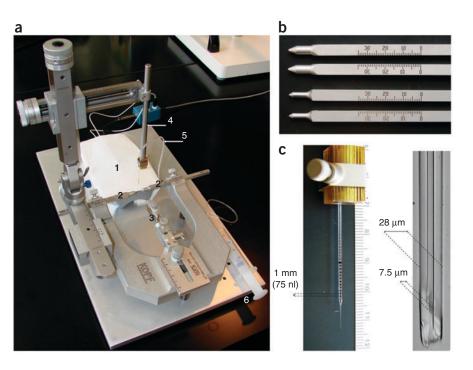


Figure 1 | Stereotaxic apparatus and the injection micropipette. (a) A typical stereotaxic apparatus: the animal is positioned on the heat-controlled blanket (1); the left (2) and right (2') ear bars and the incisor adaptor with the nose clamp (3) are used to fix the animal's head; and the micropipette holder (4) is controlled in the *x*, *y* and *z* planes by the stereotaxic arm. For our injection procedures, thin plastic tubing (5) is attached to the top of the glass micropipette in the holder (**c**, right); a 20-ml syringe (6) attached to the other end of the plastic tubing is used for aspiration and injection of the virus. (**b**) Non-rupture ear bars for use in rat (top pair) and mouse (bottom pair). The tip has a shallow angle, which prevents deep penetration of the ear and injury to the tympanic membrane. (**c**) Left, low-magnification image of the injection micropipette in the holder (with a millimeter scale at right for comparison); 1-mm marks on the shaft of the micropipette (made with a marking pen with an extra fine tip) 'calibrate' the volume in the micropipette: 1 mm = 75 nl. Right, high-magnification image of the tip of the micropipette; the long narrow tip (diameter, 7.5 μ m) helps to inject the virus into the brain parenchyma, and the sharp edges at the tip help to easily penetrate the dura in mice and young rats.

• Surgical sutures (silk)

• Temperature-controlled heating blanket (e.g., FHC) or heat lamp **REAGENT SETUP**

Purified rAAV or lentivirus Viruses are resuspended in phosphate-buffered saline (PBS); we typically use a titer of at least 1×10^6 of infectious particles per microliter, although a lower titer may be advantageous for sparse infection. **! CAUTION** The rAAVs and lentiviruses are infectious reagents with a biosafety level of BL-2 and should be handled according to relevant institutional guidelines; protective gloves and a lab coat should be worn when handling the virus stock (NIH guidelines available at http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm).

EQUIPMENT SETUP

Micropipettes for injections We use 5-µl calibrated micropipettes (VWR or Blaubrand); these are first 'pulled' on a micropipette puller (such as Sutter P-97) to create a long narrow shank, which is then clipped with scissors to create a tip opening of about 7–9 µm (a smaller tip will lead to clogging; a larger tip will lose the ejection pressure achieved with a tip of less than 9 µm, causing some virus to seep back to the surface along the trajectory of the needle); the micropipette shaft is marked with a 1-mm scale, where 1 mm corresponds to a volume of 75 nl (**Fig. 1**).

PROCEDURE

Preparation of surgical area and tools

1 Before starting the surgery, ensure that the surgical area and all tools and reagents are clean and ready to be used during the procedure. The area should be disinfected by wiping with 70% ethanol and the tools should sterilized either by autoclaving or by immersion in disinfectant. In addition to standard surgical tools, we create 'home-made' surgical hooks from clipped and bent needles (described below).

Anesthesia and fixation of animal in stereotaxic apparatus • TIMING less than 10 min (less than 20 min with pre-anesthesia)

2| Weigh the animal and calculate the appropriate dose for pre-anesthesia (optional) and anesthesia. We use a mixture of ketamine and xylazine at a dose of 80–100 mg ketamine and 10 mg xylazine per kilogram body weight, given intraperitoneally, for adult mice and rats, and 40–50 mg ketamine and 5 mg xylazine per kilogram body weight, given intraperitoneally, for P7–P10 pups. We always use pre-anesthesia with atropine, at a dose of 0.02 mg per kilogram body weight, given subcutaneously, for young animals (mice and rats between P7 and P14), to reduce bronchial secretion and improve breathing. To inject atropine, gently restrain the animal and inject it subcutaneously in the scruff at the back of its neck.

3 After injecting atropine, wait 10 min before injecting the anesthetic.

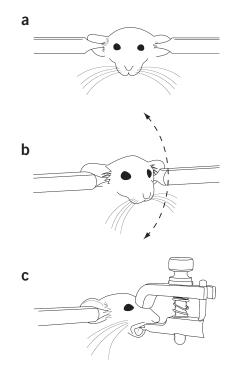


Figure 2 | Correctly positioned and fixed mouse head. (a,b) Fixation with ear bars. The front view (a) shows the symmetrical positioning of the head; the rotated view (b) shows that the nose is able to move freely up and down, but lateral movement is constrained by the fixation. (c) After insertion of the incisor adapter, the tongue is gently pulled out and aside to prevent breathing problems. The nose clamp is gently positioned or can be omitted altogether.

4 To inject the ketamine-xylazine mixture, restrain the animal with one hand (for mice and young rats) or in a plastic cone (for larger rats) with abdomen facing up and inject the ketamine-xylazine mixture intraperitoneally through a needle inserted into the lower left abdominal quadrant (needle size, 22–23 G). The animal should fall asleep (but still be sensitive to nociceptive stimuli) within 2–3 min and should reach surgical anesthesia (lack of response to nociceptive stimuli) within 5–10 min.

? TROUBLESHOOTING

5|Shave the fur on the skull, clean the skin with 70% ethanol and place the animal in the stereotaxic apparatus (**Figs. 1** and **2**). To place the animal in the apparatus, fix one ear bar in the apparatus, gently position the animal's head to lead its ear canal onto the ear bar, keep the animal's head in place and slowly position the second ear bar to complete the fixation (apply only very moderate pressure).

▲ **CRITICAL STEP** Because this procedure involves recovery from surgery, use only non-rupture ear bars with wide angle tip to avoid injuring the tympanic membrane (**Fig. 1**); avoid any quick movements when positioning the ear bars. When done correctly, the animal's head looks horizontal and symmetrical to the ear bars (**Fig. 2a**); the head can be freely moved up and down by gently moving the nose, but will not move laterally with respect to the ear-bar axis (**Fig. 2b**). ▲ **CRITICAL STEP** Apply lubricant eye ointment to prevent corneal drying during the surgery.

6 Insert the incisor adapter. Use small forceps to pull down the animal's lower jaw, slowly move the incisor adapter into the animal's mouth until the animal's incisors 'fit' in the opening of the adapter, then gently pull back slightly and fix the adaptor in place (small back traction on the animal's incisors improves head fixation). The last point of fixation, the nose clamp, should be used with very low pressure on the animal's nose. Make sure that the clamp does not reach the eyes, as may happen with young animals (we often omit the nose clamp altogether, using only the ear bars and the incisor adapter for fixation). Finally, gently pull the tongue out and aside with small dull forceps (**Fig. 2c**); this helps to prevent breathing problems, especially in young animals. **? TROUBLESHOOTING**

Surgery (preparation of craniotomy) TIMING 15–20 min

7 Use a dissecting microscope at a low magnification ($\times 10$ to $\times 20$) to visualize the top of the animal skull. Make a midline incision with small surgical scissors or scalpel. Separate the subcutaneous and muscle tissue and use small surgical hooks to keep the area open (Fig. 3). Gently clean the bregma and lambda areas (Box 1, Fig. 4) using a small bone scraper. Level the head of the animal by measuring the z coordinates of bregma and lambda and adjusting the head position so that they become equal (in most stereotaxic apparatuses this can be easily done using an adjustment screw at the incisor adapter). Then level the head horizontally in the caudal-to-rostral direction (leveling in the medial-to-lateral direction is achieved by proper positioning of the

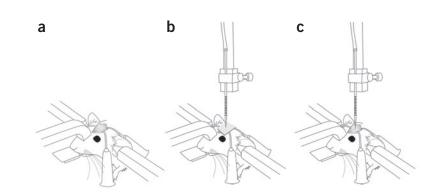


Figure 3 | Surgery and delivery of the virus. (a) The surgical wound is kept open by short needles with blunted tips bent to form small hooks; the skull is kept moist with PBS during the surgery. (b) After the craniotomy is drilled, virus (a $4-\mu$ l drop on parafilm positioned atop the skull) is aspirated into the micropipette by suction with a 20-ml syringe attached by thin tubing; aspiration of the virus in the shaft of the micropipette is monitored with a dissecting microscope. (c) After the micropipette is positioned in the brain parenchyma at the desired *x*, *y* and *z* coordinates, the virus is injected by slow pressure with a hand-held syringe. The speed and volume of the injection is monitored as the top level of the virus solution moves across the calibration marks on the micropipette, with 1 mm corresponding to 75 nl of injected volume.

ear bars in the animal ear canals, described above).

▲ CRITICAL STEP Keep the skull moist with sterile PBS (or saline), applied as drops or with a wet cotton swab, throughout the surgery.

8 Measure the position of the *x* and *y* coordinates of bregma and calculate (subtract) the coordinates of the target injection area, as determined from a stereotaxic brain atlas (**Box 1**, **Table 1**) examples of stereotaxic coordinates for adult mouse brain).

9 Thin the skull over the target area (about $1 \text{-mm} \times 1 \text{-mm}$ for one injection site) using a hand-held drill by horizontal movement of the spinning drill bit while applying a slight pressure downward. We typically use ×40 magnification of the dissecting microscope during drilling. Stop when the bone is very thin (blood vessels in the dura become clearly visible; sometimes a crack appears in the thinned bone).

▲ CRITICAL STEP Do not drill through the bone, as that will probably cause an injury to the surface of the brain parenchyma. Take a small needle (27 G) and carefully perforate the edges of the craniotomy; next, with the tip of the needle, 'flip up' a piece of the thinned bone and then carefully remove it with fine (#5) forceps. Keep both the skull and exposed dura moist with PBS.

Injection of virus TIMING 10–15 min for a single injection

10| Place the injection micropipette (**Fig. 1**) into the holder of the stereotaxic arm and connect thin plastic tubing at the top end. Pipet a 4- μ l drop of virus onto a precut square of parafilm (about 1 cm \times 1 cm) and place the parafilm with the virus on top of the skull (**Fig. 3b**).

! CAUTION All material that comes in contact with the virus should be handled as BL-2 biosafety waste, collected in special containers and disposed of only after being autoclaved or inactivated with bleach or other disinfectant; wear protective gloves and a lab coat.

Bring the tip of the micropipette into the drop of the virus using visual control with the dissecting microscope; attach a 20-ml syringe to the end of the tubing and apply suction. Visually monitor with the dissecting microscope the rising level of the virus in the micropipette shaft. The achieved of the

the virus in the micropipette shaft. The aspiration of the virus should be very slow with a tip opening of less than 9 μ m, even with application of full suction with the syringe. Continue suctioning until the desired volume of virus is aspirated (measure volume using the calibration marks on the micropipette, where 1 mm = 75 nl; **Fig. 1**). When done, unplug the syringe from the tubing to release the suction and only then move the tip of the micropipette above the parafilm. Remove the parafilm and place it in the BL-2 waste container.

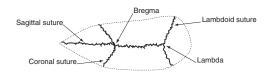


Figure 4 | Stereotaxic landmarks on the skull. Rodent skull surface diagram includes the sagittal, coronal and lambdoid sutures defining the stereotaxic landmarks bregma and lambda.

11| Bring the tip of the micropipette to bregma and calculate the *x*, *y* and *z* coordinates that will be used for the injection. Bring the micropipette to the correct *x* and *y* position and lower it until it touches the exposed dura. The cut tip of the micropipette often has a sharp edge (**Fig. 1c**), which allows easy penetration of the dura of mice and young rats. If the tip does not penetrate easily (which always occurs in rats older than 2 weeks), cut a small incision in the dura with a 27-G needle held at a flat angle (avoid injuring the brain surface by pointing the needle downward).

▲ CRITICAL STEP Be careful not to push the dura too strongly downward while trying to penetrate it; doing so will cause a contusion on the brain surface.

After penetrating the dura, slowly lower the micropipette to the desired z coordinate of the injection site.

12 Attach the 20-ml syringe to the end of the injection tubing, focus the dissecting microscope on the level of the viral solution in the capillary and begin to slowly apply pressure with the syringe to inject the virus. Control the speed and volume of the injection by visually monitoring the movement of the virus solution in the micropipette shaft across the calibrating marks. Because of the small opening of the long tip of the micropipette (**Fig. 1c**), low, steady pressure with the syringe typically results in a slow rate of 75 nl (1 mm across the shaft) per minute; however, it is possible to inject at an even slower rate. When finished, unplug the syringe from the injection tubing.

? TROUBLESHOOTING

13 Wait 2–3 min before withdrawing the micropipette; then withdraw slowly to avoid backflow of the virus to the surface. Dispose the micropipette into an BL-2 waste container for glass.

Clean the injection site with moist cotton swabs (dispose of these as BL-2 waste). A small craniotomy (less than $1 \text{ mm} \times 1 \text{ mm}$) does not need to be covered with bone wax; for a larger craniotomy, apply a thin 'slip' of bone wax over the skull (be careful not to push the bone wax downward, as that may cause contusion of the brain surface).

▲ **CRITICAL STEP** Suture the skin and apply triple antibiotic ointment to the wound. Inject the anesthetic lidocaine subcutaneously near the wound for local anesthesia during the early recovery period. Inject sterile PBS (30 ml per kilogram body weight) subcutaneously to avoid dehydration of the animal after the surgery.

Recovery from anesthesia • TIMING 10-30 min, depending on the time of the last injection of ketamine-xylazine during surgery

14 Keep the animal warm (either on a temperature-controlled heat blanket or under a heat lamp) until it fully recovers. Once the animal recovers, inject the analgesic buprenorphine at a dose of 0.05 mg per kilogram body weight. Return the animal to a clean cage (single-housed); put a small dish with wet food pellets in the cage for easy access to food. Inject additional analgesic (meloxicam at a dose of 1 mg per kilogram body weight) on the first and second days after the surgery. Monitor the recovery of the animal closely for at least 1 week, assessing any signs of distress, such as piloerection, lack of grooming, reduced locomotion, wound scratching and inflammation of the surgical wound.

15| The time of recombinant *in vivo* expression from the rAAV or lentiviral vectors will vary, depending on the experimental design, from only days to weeks or even months. For example, 4–5 d may be enough to achieve sufficient GFP labeling of neurons for targeted patching of *in vitro* in brain slices, but more time may be needed to achieve the desired experimental manipulation, such as gene 'knockdown' through coexpression of short interfering RNA²⁶. Anatomical labeling by expression of GFP, especially for long-distance axonal tracing, may require more expression time, typically 1–2 months²⁷.

• TIMING

Steps 1-6 (anesthesia and stereotaxic fixation): 10 min (20 min with atropine pre-anesthesia) Steps 7–9 (preparation of craniotomy): 15–20 min Steps 10-13 (brain delivery of the virus): 10–15 min per single injection Step 14 (recovery from anesthesia): 10–30 min

? TROUBLESHOOTING

Animal not anesthetized after 10 min

There is some variability in the response to ketamine-xylazine, and this may require an increase in the dose: inject an additional 20–25% of the dose intraperitoneally and wait 5–10 min; if the animal is still only lightly anesthetized, repeat with 10–12.5% of the dose.

Animal struggles with breathing

Bronchial secretions may cause breathing problems (in such cases, the breathing may seem laborious and/or the animal may make small 'wet' sounds). Release the animal from the stereotaxic apparatus and ensure its tongue is pulled out;

place the animal in your palm and gently enter the animal's mouth and upper trachea with thin tubing attached to a 3-ml syringe and slowly aspirate. Provide 100% oxygen.

Animal stops breathing

Use the same procedure described above; gently compress thorax between thumb and forefinger at a high rate (about 100 per min); put a drop of atropine on the animal's tongue.

Animal becomes insufficiently anesthetized

If the animal begins breathing more rapidly and/or has lightly active pedal reflex but is otherwise asleep, inject an additional 25% dose and closely monitor if the anesthesia deepens. If the animal begins to awaken (the first muscle movements are rapid twitches of the large facial whiskers), quickly release the animal from the stereotaxic apparatus and then re-anesthetize the animal.

Micropipette clogs

Raise the micropipette and rinse it in a large drop of PBS on parafilm, alternating suction and ejection to clean the tip; if it remains clogged, replace it with a new micropipette (do not cut the tip with scissors, as that will make the tip much larger and injection will cause backflow of the virus onto the brain surface along the trajectory of the micropipette).

ANTICIPATED RESULTS

Stereotaxic gene delivery by recombinant viruses in the rodent brain is a highly versatile technique applicable to most (if not all) research areas of cellular neuroscience. This approach has a distinct advantage over work with cultured cells, as the *in vivo*-infected neurons retain their native properties, minus the introduced genetic manipulation, in otherwise intact neuronal networks. The method also has some advantages over traditional mouse genetics, including ease of application, high spatiotemporal control over the introduced manipulation and a short time between experimental design and data collection. In addition, as only a small population of neurons is affected, it is possible to use this approach for studies of gene functions that would otherwise result in lethality or in the activation of compensatory mechanisms if the entire brain or large brain regions were altered. The main disadvantage of the technique, so far, is the limited possibility for cell type-specific manipulations; in addition, if large brain regions must be altered, e.g., for study of gene functions in complex behavior, mouse genetics is the better suited approach.

Finally, as the protocol described here involves recovery from surgery, we must emphasize the absolutely critical importance of taking proper care of the animals both during and after the procedure. For more information about rodents, their coexistence with humans throughout history, and their great contributions to science and the treatment of human disease, we recommend visiting the Rodent Respect website (http://www.rodentrespect.com); in our own laboratory, that is 'homework' for all students beginning work with rats and mice.

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

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Erratum: Stereotaxic gene delivery in the rodent brain

Ali Cetin, Shoji Komai, Marina Eliava, Peter H Seeburg & Pavel Osten

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In the PDF version of this article initially published online, several lines of text were omitted. The last sentence on page 3167 ("Another well suited application is the use of recombinant viruses for anatomical tracing;") should have been followed by "for example, simply labeling the entire axonal arborization with GFP27. Finally, *in vivo* cellular analysis of virally infected and manipulated cortical cells is possible in anesthetized rats and mice with the two-photon targeted-patching technique²⁸⁻³⁰. As for the study of gene function in behavior, several groups have used viral vectors to infect specific brains regions in mice, such as the midbrain ventral tegmental area and lateral amygdala, and have demonstrated distinct behavioral phenotypes^{31,32}. The main advantage of this approach lies in the exceptional spatiotemporal control over the induced genetic manipulation, which avoids the confounding variables, such as gene alterations during early development and/or in multiple brain regions, typically associated with traditional mouse genetics." The error has been corrected in the PDF version of the article.