Published in final edited form as:

Methods Mol Biol. 2018 ; 1867: 229–238. doi:10.1007/978-1-4939-8799-3_17.

Stereotaxic Surgery and Viral Delivery of Zinc-Finger Epigenetic Editing Tools in Rodent Brain

Peter J. Hamilton¹, Carissa J. Lim², Eric J. Nestler¹, and Elizabeth A. Heller³

¹The Friedman Brain Institute, Icahn School of Medicine at Mount Sinai, New York, NY, USA.

²Department of Pharmacology and Penn Epigenetics Institute, The University of Pennsylvania, Philadelphia, PA, USA.

³Department of Pharmacology and Penn Epigenetics Institute, The University of Pennsylvania, Philadelphia, PA, USA. eheller@pennmedicine.upenn.edu.

Abstract

Delivery of engineered zinc-finger proteins (ZFPs) for targeted epigenetic remodeling in rodent brain can be facilitated by the use of viral vector-mediated gene transfer coupled with stereotaxic surgery techniques. Here we describe the surgical protocol utilized by our group which is optimized for herpes simplex virus (HSV) delivery into mouse brain. The protocol outlined herein could be applied for delivery of adeno-associated viruses (AAV) or lentiviruses in both mice and rats. This method allows for the viral expression of engineered DNA-binding factors, particularly engineered ZFPs, and subsequent epigenome editing in rodent brain with excellent spatiotemporal control. Nearly any brain region of interest can be targeted in rodents at every stage of postnatal life. Owing to the versatility, reproducibility, and utility of this technique, it is an important method for any laboratory interested in studying the cellular, circuit, and behavioral consequences of in vivo neuroepigenetic editing with synthetic ZFP constructs.

Keywords

Viral mediated gene transfer; Zinc-finger proteins; Neuroepigenome editing; Stereotaxic surgery; Rodent brain

1 Introduction

Stereotaxic surgery is a powerful method used to manipulate the brain of living animals. This technique allows researchers to consistently and accurately target deep structures of the rodent brain through the use of a stereotaxic brain atlas, which provides the coordinates of a given brain area relative to Bregma, an anatomical landmark on the rodent's skull. Stereotaxic coordinates for rodent brain regions of interest can be determined from *The mouse brain in stereotaxic coordinates* [1] and *The rat brain in stereotaxic coordinates* [2]. Facilitated through the use of a stereotaxic instrument, one can perform this surgery on large numbers of anesthetized animals to reliably and accurately access structures within the rodent brain.

Combining this approach with viral mediated gene transfer, which has been widely used as a means to introduce transgenes to intact brain tissue [3], researchers have been successful in delivering engineered ZFP neuroepigenome editing tools to deposit genelocus-specific modifications in vivo to alter neural function and animal behaviors [4–6]. ZFP epigenetic editing tools, which can exogenously introduce chromatin modifications at a single genomic locus within neurons or even a single type of neuron in an injected brain region [4–8], are necessary to establish the causal relevance of such mechanisms to gene expression and neural function. Given the fact that regulation of epigenetic landscapes is central to neuropsychiatric health and disease [9, 10], it is crucial to combine epigenetic editing techniques with in vivo inquiry in the brains of awake and behaving animals. The technique of viral expression of ZFP epigenetic editing tools in rodent brain using stereotaxic surgery techniques facilitates the exploration of the causal impact of the targeted chromatin modifications in these neurobiological contexts.

2 Materials

2.1 Reagents

1. Ketamine and xylazine
2. 70% Ethanol
3. 100% Acetone
4. 10% Bleach solution
5. Alcohol prep wipes
6. Sterile ocular lubricant
7. Sterile PBS
8. Sterile normal saline
9. Purified virus (HSV, AAV, lentivirus)
10. Betadine antiseptic
11. Bupivacaine HCl local anesthetic

2.2 Instruments and Materials

1. Dual-small-animal stereotaxic instrument (such as Kopf Model 902)
2. Fine Science Surgical Tools, including but not necessarily limited to scalpel, scissors, forceps
3. Laboratory scale
4. Bead sterilizer
5. Electric hair shaver
6. Sterile tip cotton swabs
7. Biohazard bags

8. Low-binding, 0.65 mL microcentrifuge tube
9. Needles and syringes for IP injection of anesthetics and analgesics
10. Absorbent lab bench diapers
11. Handheld dental drill and 0.6 mm burr
12. Hamilton syringes (5 μ L Catalog #848:51) with Hamilton small-gauge RN needles (33-gauge Catalog #7762–06)
13. Tissue adhesive, surgical clips, or surgical sutures
14. Temperature-regulated heating pads and/or heat lamp

3 Methods

3.1 Stereotaxic Surgery

1. Position the stereotaxic instrument (*see* Note 1) under a heat lamp. Make sure that the surgical area is cleaned with 70% ethanol and surgical instruments are cleaned and properly sterilized. We find that a bead sterilizer works well for this purpose. Cover the surgical area with absorbent lab bench diapers. All procedures should be performed in accordance with your institution's biosafety and animal use guidelines.
2. Place Hamilton syringes in arms of the stereotaxic instrument and clear any blockages by drawing and expelling 100% acetone five times. Subsequently draw and expel sterile PBS five times to remove any residual acetone. Draw the maximum volume of sterile PBS into the Hamilton syringe, taking care to include no bubbles. Swing the stereotaxic arms to move the Hamilton syringes out of the way of the workspace in the center of the instrument.
3. Anesthetize animals with a ketamine/xylazine mixture (100 mg/kg ketamine and 5 mg/kg xylazine in sterile normal saline) delivered via intraperitoneal injection. The animal should reach surgical anesthesia within 5–10 min, and should not respond to a light pinch to the hind paw (*see* Notes 2 and 3).
4. Cover the anesthetized animal's eyes with sterile ocular lubricant to keep them moist during the surgery.
5. Shave the fur off of the top of the animal's skull and clean the surface of the skin with alcohol prep wipes. Apply Betadine antiseptic using sterile tip cotton swabs.
6. Place the animal in the stereotaxic instrument. To do so, carefully place one ear bar in the ear canal, secure the bar, and hold the animal in place as the other ear bar is placed and secured. The animal should not be able to move laterally. Next, secure the mouth in the incisor adapter of the stereotaxic instrument, taking care that the tongue is not pinched in the adapter or blocking the airway. The nose clamp can be gently tightened to firmly secure the animal's head in position (*see* Notes 4 and 5). Visually inspect the head and make adjustments to the pitch of the incisor adapter to make sure that the head is level (Fig. 1a).

7. Make a midline incision to the top of the animal's skull with small surgical scissors or a scalpel. Use small surgical clips to gently keep the incision open, providing access to the skull. Optionally, sterile saline can be used with sterile swabs to clean the skull to aid in visualization of stereotaxic landmarks on the skull (Fig. 2).
8. Measure the z coordinates of Bregma and Lambda on the animal's skull and adjust the position of the head with the incisor adapter until they become equal. This serves to level the skull. Adjust the pitch of the ear bar to ensure that the skull is completely flat.
9. Position the tip of the Hamilton syringes to Bregma and record the x , y , and z coordinates on the vernier scale located on the arms of the stereotaxic instrument. Subtract the coordinates of the targeted brain region to calculate the site of targeted viral injection. These coordinates can be determined from a stereotaxic brain atlas (*see* Subheading I and Note 6). Note that the angle of the stereotaxic arm is an important consideration when determining the coordinates for targeting a desired brain region.
10. Position the tip of the Hamilton syringes according to the calculated x and y coordinates. Using a dental drill with a 0.6 mm burr, thin the area of the skull directly under the Hamilton syringe tip. Do not apply excessive downward force, as it may result in drilling through the skull and damaging the surface of the brain. Lower the Hamilton syringe on the z coordinate until it slides through the thinned skull, and raise the Hamilton syringe above the surface of the skull.
11. Proper safety attire and handling techniques should be applied based on the biosafety level of the virus being used (*see* Note 7). Defer to your institutional biosafety requirements for proper safety attire and handling techniques. The use of HSV vectors for our ZFP-mediated epigenome engineering experiments necessitates the use of a lab coat, gloves, and goggles when handling the virus. Place a viral aliquot in a low-binding, 0.65 mL microcentrifuge tube on wet ice, allowing it to thaw.
12. Taking care not to alter the x or y coordinates, expel 2.5 μ L of sterile PBS from the Hamilton syringe. This volume will accumulate on the tip of the syringe, indicating unobstructed flow through the syringe tip (*see* Note 8). This volume can be removed with a sterile tip cotton swab. Draw the plunger up by an increment of 0.5 μ L to introduce a small air bubble into the barrel of the syringe. This serves to separate the viral solution from the sterile PBS. Finally, pull up the desired volume of virus to inject (typically 0.5–1 μ L) and place the microcentrifuge tube back on wet ice.
13. Slowly lower the Hamilton syringe through the burr hole in the animal's skull to the calculated z coordinate to the desired injection site within the brain (Fig. 1b).
14. Deliver the viral solution by lowering the plunger of the Hamilton syringe at a rate of 0.1 μ L per minute. Once the full volume of the viral solution has been dispensed, wait for 5 min for the virus to diffuse through the tissue (*see* Note 9).

15. To avoid backflow of the virus to the surface of the brain, *slowly* raise the Hamilton syringe out of the skull.
16. Expel the remaining contents of the Hamilton syringe into a flask containing a 10% bleach solution and use an alcohol prep wipe to remove any material that may have accumulated on the syringe tip.
17. Remove the animal from the stereotaxic instrument and close the incision via surgical suture or tissue adhesive. Small burr holes (less than 1 mm in diameter) do not need to be covered with bone wax. Apply antibiotic ointment to the wound and inject the local anesthetic bupivacaine subcutaneously near the wound, to reduce discomfort during the recovery period.
18. Place the animal in a clean cage that is warmed either by a temperature-regulated heating pad or a heat lamp until the animal fully recovers. This should take approximately 20 min, depending on the duration of the surgery.
19. Return the animal to a clean cage with moistened food pellets for easy access to food. Monitor the animal's recovery, looking for any signs of distress which can include a lack of grooming, wound scratching, inflammation, altered locomotion, or reduced weight gain.
20. Clean the Hamilton syringes with 100% acetone and sterile PBS and according to the manufacturer's instructions (*see* Note 10). Discard the lab bench diapers into a biohazard receptacle and clean the workspace with 70% ethanol.
21. The time to maximal *in vivo* expression of our HSV-delivered, engineered transcription factors is approximately 2–3 days and persists through days 8–10 (Fig. 3). During this window, any number of molecular or behavioral experiments can be performed.

3.2 Validation of Neuroepigenomic Editing Tools

It is essential to validate the use of chromatin-modifying tools in several ways to ensure their effectiveness and selectivity *in vivo* [4, 5, 11]. Depending on your application, the essential validations may vary. The outline below provides a general list of suggested validations.

1. Validate expression of the tool (e.g., a fusion of a chromatin-modifying moiety to ZFP) in the brain *in vivo* (*see* Note 11). This includes validating selective expression in neurons when using a neurotrophic vector like HSVs, as well as selective expression within a single type of neuron if using a Cre-dependent vector in a mouse line that expresses Cre recombinase in a given cell type.
2. Validate that the epigenetic editing tool produces the designated chromatin modification at the targeted locus, for example, that the -p65 effector domain induces histone acetylation, that -G9a induces H3 Lys9 dimethylation, that -Tet1 induces DNA hydroxymethylcytosine, and that -Dnmt induces DNA methylation.
3. Determine whether the designated epigenetic modification is associated with altered expression of the targeted gene. This could be performed through the

utilization of quantitative PCR, Western blot analysis, or other techniques to quantify expression levels of the product of the targeted gene. This analysis demonstrates that the targeted, ZFP-mediated epigenetic reprogramming at a given locus is sufficient to regulate gene expression through a physiologically relevant mechanism.

4. Study whether the designated chromatin modification is associated with other forms of epigenetic regulation, transcription factor binding, or changes in chromatin architecture. This line of inquiry is important to convey that the targeted epigenetic editing is capable of functionally integrating into the endogenous biological mechanisms of epigenetic regulatory events. These analyses could be performed with chromatin immunoprecipitation (ChIP) of transcription factors or epigenetic marks in question followed by quantitative PCR or next-generation sequencing, among other approaches.
5. Validate that the neuroepigenomic editing tool is selective for the targeted locus (*see* Note 12).

4 Notes

1. Ensure that the stereotaxic frame and accessories including the ear bars and incisor adaptor are appropriate for the type of animal to receive surgery.
2. If the animal does not reach a sufficient level of surgical anesthesia after 10–15 min, inject an additional 20% dose of ketamine/xylazine. Closely monitor the animal to confirm that the anesthesia deepens.
3. If the animal begins to awaken during surgery, remove the animal from the stereotaxic instrument and reapply the anesthesia. The early signs of an animal awakening from anesthesia include twitches of the large facial whiskers and twitching of the tail. With careful monitoring, this occurrence can be avoided.
4. It is essential that the animal be firmly secured in the ear bars. Visually validate that the ear bars arc in the ear canal and not pinching the jaw, neck, or skull. Animals appropriately positioned in the ear bars will be able to move their snout up and down in the incisor adapter, but will not be able to move side to side.
5. If the animal is not securely placed in the stereotaxic instrument, then it is possible that the skull's position will shift when drilling burr holes. This invalidates all recorded coordinates. To be sure this does not occur, when the animal is first positioned in the instrument; apply light pressure to the skull with a sterile tip cotton swab. If the animal's skull shifts in response to the pressure, resecure the animal within the stereotaxic instrument.
6. The stereotaxic coordinates provided in atlases are optimized for adult, male animals. If experiments involve varying from these average metrics, it becomes important to validate and/or alter targeting coordinates through pilot experiments. In short, use the stereotaxic atlas coordinates as initial values,

perform surgeries, and validate viral targeting with fluorescent microscopy. Adjust the stereotaxic coordinates as needed.

7. Selecting the appropriate viral vector for delivery of neuroepigenomic editing tools is paramount to the success of these experiments. Each viral vector varies in its spread, packaging capacity, tropism, transgene expression timing, and duration of expression. These variables should be carefully considered, and pilot studies should be performed to empirically validate viral function.
8. If the Hamilton syringe clogs, it will prevent dispersion of the virus into the brain. Always visually confirm that the flow from the syringe is not impeded by expelling a very small volume of the viral solution back into the microcentrifuge tube before lowering into the rodent brain. If the syringe does not appear to work, expel the contents of the syringe into a 10% bleach solution, clean with 100% acetone and/or replace the Hamilton needle, and restart the process of loading viral solution into the Hamilton syringe.
9. The Hamilton syringe tip is left in place during the 5-min rest after delivering virus in order to prevent backflow of viral solution up the needle track. However, we have found it beneficial during this time to *slightly* retract the Hamilton syringe along the *z*-axis (<1 mm) to provide a small space for the more even dispersion of the viral solution in the tissue.
10. If using surfactant-based cleaning solutions for cleaning and maintaining Hamilton syringes (as is often suggested according to the manufacturer's instructions), be extremely vigilant to thoroughly remove all traces of soap, as it can be damaging to viral function and titers. There should be no formation of soap bubbles when pipetting sterile PBS.
11. Introducing an epitope tag onto the neuroepigenome editing tool facilitates more convenient immunohistochemical approaches for validating expression, among other uses.
12. To date, validation of selectivity of targeting has been accomplished primarily by identifying regions of the genome that are most highly homologous to the targeted region and demonstrating lack of changes of chromatin modifications of these other regions and lack of altered expression of any nearby genes. Ultimately, it is advantageous to demonstrate direct and selective binding of the epigenetic editing tool to the targeted locus and to no other genomic location. This has been successfully performed in cultured cell lines [11, 12]. However, this is challenging, particularly in brain *in vivo*, since every cell only has two specific binding locations and the quantity of infected tissue can be limiting.

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Fig. 1. Correct placement of rodent's head within stereotaxic instrument and surgical procedure for viral delivery. **(a)** A cartoon depicting the fixation of animal's head within the stereotaxic instrument. The ear bars are securely in place, preventing lateral movement of the skull. The incisor adapter restricts vertical movement, with the nose clamp gently tightened into place. **(b)** Upon surgically exposing the stereotaxic landmarks on the skull, the stereotaxic coordinates are measured relative to Bregma. Hamilton syringes are used to deliver the viral solution to desired regions within the animal's brain via small burr holes in the animal's skull

Fig. 2. Stereotaxic landmarks on the skull. The diagram above depicts the stereotaxic landmarks Bregma and Lambda on the exposed surface of the rodent's skull

Fig. 3.

Stereotaxic delivery of HSV to discrete brain regions. HSV expressing GFP under the control of the CMV promoter was stereotaxically injected into the nucleus accumbens (NAc) of a mouse to demonstrate the transduction efficiency and spread of the HSV viral vectors (image previously published [13]). These viral vectors are capable of co-expressing neuroepigenome editing constructs under the control of distinct promoters. The injection was performed at a 10^0 lateral angle at +1.6 anterior/posterior, +1.5 mediolateral, and -4.4 dorsal/ventral coordinate relative to Bregma. Scale bar is 300 μ m