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Use of Adeno-Associated and Herpes Simplex Viral Vectors for *In Vivo* Neuronal Expression in Mice

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Abstract

Adeno-associated viruses and the herpes simplex virus are the two most widely used vectors for the *in vivo* expression of exogenous genes. Advances in the development of these vectors have enabled remarkable temporal and spatial control of gene expression. This unit provides methods for storing, delivering, and verifying expression of adeno-associated and herpes simplex viruses in the adult mouse brain. It also describes important considerations for experiments using *in vivo* expression of these viral vectors, including serotype and promoter selection, as well as timing of expression. Additional protocols are provided that describe methods for preliminary experiments to determine the appropriate conditions for *in vivo* delivery.

Keywords

adeno-associated virus; herpes simplex virus; *in vivo*; brain; mouse

INTRODUCTION

This unit provides a basic protocol for introducing either adeno-associated or herpes simplex virus into the mouse brain. Recombinant adeno-associated viral vectors (AAVs) and herpes simplex viral vectors (HSVs) are the most commonly used vectors in the neuroscience literature as a mechanism for expression of exogenous genes in the adult brain. Recent advances in vector development allow transgenes to be delivered efficiently *in vivo* in a cell type-specific and temporally precise manner. In this unit, protocols for sub-aliquoting, delivery, and validation of AAV and HSV in the adult mouse are provided. Basic Protocol 1 details the aliquoting and storage of vectors. Basic Protocol 2 outlines delivery of virus into the brain of an adult mouse. Finally, Basic Protocol 3 describes validation of AAV or HSV expression and confirmation of placement in the mouse brain using immunohistochemical (IHC) and protein (western) immunoblotting techniques (see also Alternative Protocol 1). Supporting Protocol 1 is also included and discusses initial experiments to be conducted with new viral vectors to determine the appropriate infusion volume and expression time course.

BASIC PROTOCOL 1

Preparation of aliquots and storage of viral vectors for use *in vivo*

This protocol details the process of preparing sub-aliquots of previously produced AAVs or HSVs for storage prior to use *in vivo*. Sub-aliquoting into smaller, practical volumes

minimizes the likelihood of repeated freeze-thaw cycles that can damage the quality of the virus preparation.

Materials—Prepared virus (AAV or HSV)

Typically provided by a core facility or company in a glycerol/phosphate buffered saline (PBS) solution. No additional dilution is required unless previously determined for your virus of interest. For optimal performance *in vivo*, viruses should be provided at a minimum titer of 10^{11} genome copies (GC)/ml and 3×10^8 infectious units (IU)/ml for AAV and HSV, respectively. There are a variety of titrating methods, each with an associated unit (*e.g.*, GC/ml, IU/ml, viral particles/ml, multiplicity of infection). A range of titers can produce sufficient transduction; however, experimental considerations such as brain region, promoter selection, and cellular tropism can influence transduction independently of titer. For best practice, each viral preparation should be tested empirically to determine the required titer for optimal transduction.

Wet Ice

Low binding microcentrifuge tube (0.65– 0.7 ml)

e.g., Corning Low Binding Plastic Microcentrifuge Tubes

Pipetman and filter tips of appropriate volumes (0.2– 2 μ l)

Tube rack $\times$ 2

One standing rack for tube holding inside the hood, one floating rack for flash freezing

Ethanol-resistant lab pen or marker

Dry Ice/ethanol tub for flash freezing

Use layer of dry ice covered by 100% ethanol to produce super-cooled ethanol suitable for flash freezing aliquot tubes.

Tabletop microcentrifuge (low-speed)

NOTE: Sub-aliquoting is conducted in a BSL-2 hood dedicated to viral work to maintain a clean workspace and keep viral constructs separate from other experiments. AAVs are considered BSL-1 by most safety committees, whereas HSVs are almost always considered BSL-2. Safety risks can be further minimized by always wearing personal protective equipment when handling virus. This includes gloves, lab coat, hair covering, shoe covering, protective eyewear, and a mask. Consult your institution's requirements for working with these viruses and follow any recommendations.

Sub-aliquoting virus from stock

1. Thaw one tube of virus stock at a time on wet ice.

Work quickly to avoid leaving virus on ice for long periods of time. Avoid vortexing. Flick to mix and spin down using a low speed, tabletop microcentrifuge.

2. While virus is thawing, prepare low binding tubes. Label tubes clearly using an ethanol-resistant pen, and leave open in the hood.
3. Use aerosol-resistant pipet tips to sub-aliquot virus. The volume of virus should be determined based on the usual number of mice that can be completed in a single surgery session. The goal is to produce aliquots that will be entirely expended at the end of the session. This minimizes repeated freeze-thaw cycles and ensures that the same lot of virus is used in each set of surgeries.

Appropriate infusion volumes will need to be determined experimentally for each brain region and virus. Assume nearly complete recovery of the aliquot if using proper syringe loading techniques and low binding tubes.

4. Flash freeze sub-aliquots by placing in a partially submerged tube rack in the dry ice/ethanol bath.
5. Store sub-aliquots at -80°C until use. On surgery days, maintain aliquots at -20°C or on ice until immediately before use. Avoid thawing unnecessary aliquots.

In general, it is recommended that any unused virus be disposed of properly after use. Consult your institution's requirements for disposal of BSL-2 agents. For low titer aliquots, re-freezing at -20°C for re-use is not recommended. However, it is possible to subject high titer aliquots to a single re-freeze with no appreciable decline in infectivity.

BASIC PROTOCOL 2

Stereotaxic surgery for delivery of virus to mouse brain

Stereotaxic surgical delivery of AAV or HSV allows for region-specific viral transduction and exogenous gene expression. Coordinates for many regions of interest in the adult mouse brain have been published or can be determined empirically using brain atlases. A list of stereotaxic coordinates for commonly targeted brain regions is provided in Table 1. Optimal transduction of AAV in most regions usually requires infusion of 0.5– 1 μl of virus at a rate of 0.05 μl per 30 secs, followed by a post-infusion rest period. For infusions of HSV, 1– 2 μl of virus is recommended at the same infusion rate (please see Commentary – Infusion Volume for details). The slow delivery rate of small volumes enables more homogenous viral delivery and reduced tissue damage. The post-infusion rest period helps to minimize diffusion of the virus up the needle track. For larger regions, multiple infusions can be delivered to distinct targeting sites. For all *in vivo* infusions, high titer viruses should be infused to ensure maximal transduction. This protocol focuses on hand-driven infusion of virus using Hamilton syringes mounted on a universal holder arm. Other previously published protocols address pump-driven infusions, automated bregma technology, and mounted stereotaxic drilling. The following protocol is described for a standard line of mice (C57/Bl6), but it can be used for any mouse line of interest.

NOTE: All protocols using live animals should be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC). Surgeries should only be performed by trained personnel and should follow IACUC and other institutional regulations pertaining to animal welfare, survival surgeries, and handling of recombinant DNA and viral vectors.

Materials—8- to 12-week old C57/Bl6 mice

Anesthetic: ketamine (16 mg/kg body weight)/xylazine (120 mg/kg body weight) in 0.9% (w/v) NaCl, sterile filtered and stored at room temperature. Prepare dilution to be injected at a volume of 0.1 ml/10 g body weight, intraperitoneal (IP).

NOTE: The Drug Enforcement Agency (DEA) has scheduled ketamine as a Schedule III drug per the Controlled Substances Act. Use of this agent requires registration with the DEA, licensing by your state board of pharmacy, and possible additional requirements as dictated by your institution prior to ordering and use. Please note that ketamine can cause rapid and persistent behavioral (antidepressant-like) effects in rodents that may complicate the experimental design and data interpretation.

70% ethanol swabs

Betadine solution

2% Lidocaine-HCl

Virus aliquots, stored at -20°C until use (see Basic Protocol 1)

Triple Antibiotic ointment

Ophthalmic ointment

Sterile injectable saline, pre-warmed to 37°C

25– 50 ng/kg Atropine

1.7-ml microcentrifuge tubes each filled with distilled water, 10% bleach (w/v), 70% ethanol (w/v)

Dremel drill with 0.9-mm bit

26-G needles

Stereotaxic frame outfitted with mouse nosepiece and ear bars or cups

Sterilized surgical instruments

Scalpel

Forceps, blunt and fine

Small bulldog clips

Sharp scissors, ear punch, toe-clip or other equipment for animal identification

Sterile gauze

Sterile cotton swabs

5 μ l, Model 85 Hamilton Microliter Syringe with reinforced plunger

30- 33-G small hub removable needle, 1.5 in. length

NOTE: When targeting some brain regions (*e.g.*, prefrontal cortex), metal needles can produce significant tissue damage. For delicate or small regions, consider using glass pipettes. When using glass pipettes, it is important to slow the infusion rate considering that a constant rate through an injector with a smaller bore results in elevated hydraulic pressure at the injection site.

Small animal clipper

Heating pad

Holding cages with paper towel lining

Clean housing cage with fresh bedding

1-ml syringes with 26- or 30-G needles for drug administration

Sutures or #7 wound clips and holder

Vetbond or other approved adhesive

Cleaning wire 0.00350 in. O.D. to clean 31 G needle

Heat sterilizer

Animal surgical tracking form

Each form should include demographic information about the mouse (weight, identifier), coordinates (for both confirming orientation and final infusion coordinates), volume of virus injected, and post-op notes.

NOTE: Prior to infusions, coordinates and infusion volume should be verified. See Supporting Protocol 1 for pilot experiments.

Prepare the working area—*NOTE: A photograph depicting the recommended surgical setup is provided in Figure 1.*

1. Set up the stereotaxic frame in an aseptic surgical field.
2. Secure syringes to the universal holder arm.
 - 2.1. Orient syringe so that the numbers on the display are facing forward.
 - 2.2. Set the syringe so that it is parallel with the holder arm.

firmly against side of skull. If cups are affixed too low on the head, the mouse's breathing will become irregular. Adjust height on the head stage. Tighten ear cups, keeping the mouse's head straight in the head stage.

- 5.2. Ensure that the mouse is firmly placed in the head stage by pressing down gently on skull. Clean the skin with Betadine followed by a 70% ethanol wipe, and apply sterile ophthalmic ointment to the eyes.
- 5.3. Inject a small volume of 2% lidocaine under the surface of the scalp, and make a small incision to expose the skull.
- 5.4. Clear the area of tissue using sterile cotton swabs. If needed, further expose the skull surface by attaching bulldog clips to either side of the incision to pull skin away.
- 5.5. Take initial anterior/posterior (A/P), dorsal/ventral (D/V), and medial/lateral (M/L) measurements at bregma (the rostral suture intersection) with the arms at 0°, unless otherwise specified (see Table 1 for list of commonly used stereotaxic coordinates). Avoid roughly contacting the skull, which can bend the needle. Lower the needle tip slowly and take measurements when the needle tip first makes contact with the skull.
- 5.6. Confirm the mouse is placed flat in the stereotax by making comparison measurements to bregma. Advance the needle 1 mm in the anterior direction, and re-measure D/V by lowering the needle to the surface of the skull. Next, move the needle 1 mm to in the posterior direction, and re-measure D/V. If these measurements are more than 0.1 mm different from one another, adjust the height of the nose bar to change A/P angle and repeat process. If adjustments to the nose bar do not correct the difference, re-mount the mouse and re-measure.
- 5.7. If ear cups are used, complete the process described in 5.6 for the M/L axis. This is not necessary if ear bars are used. The accuracy of viral infusions critically depends on proper placement of the mouse in the stereotax frame. Natural variation in skull shape can make it difficult to have mice perfectly placed, and depending on the coordinates and region to be targeted, it is possible that greater measurement differences can be tolerated. This should be determined empirically with dye infusion experiments.

6. Perform Infusion

- 6.1. If needed for infusion target, adjust the angle of stereotax arm(s) and re-measure bregma. Once bregma has been measured it is very important to avoid disrupting the stereotax arms (by switching angle or rotating arms) in order to ensuring proper targeting.

Some coordinates are determined from the surface of the dura or pia matter, as opposed to the surface of skull. Confirm how coordinates

are derived and if needed, take measurements and conduct calculations from the brain surface. See Table 1 for additional detail. Published coordinates may not explicitly detail if coordinates are derived from skull or brain surface. Pilot dye infusion experiments should be conducted to validate all new coordinates prior to viral surgeries.

- 6.2.** Using animal surgery log, calculate the final coordinates of stereotax arms by performing calculations from the reference measure to reach targeting coordinates. Move stereotax arm(s) to calculated coordinates, lower needle, and mark skull. Skull can be marked by releasing a small volume of water from the syringe on to a dry skull, using a china pencil, or marker.
- 6.3.** Make small holes through the skull and dura mater at coordinates. A sharp, clean burr is typically used. A 26-G needle may also be used by gently pressing down on the skull and gently twisting until the skull is pierced. This method typically results in smaller diameter holes but requires more caution. Regardless of the method, take precaution to avoid excessive damage to brain when making holes.
- 6.4.** Ensure the drill holes are clear by lowering syringe. Monitor for deflections, the needles should pass cleanly through the hole. Raise syringe to working height for virus loading.
- 6.5.** Gently loosen the plunger nut to allow controlled plunger movement by turning/pressing the plunger. Eject excess water into sterile gauze or ethanol swab, leaving behind at least 1– 2 μ l. Examine ejected water to ensure it is being evenly ejected and bubble free.
- 6.6.** Load 1- 2 μ l of air into the syringe by gently turning and pulling up the plunger. The air/water interface serves as the proxy for virus/air interface when tracking infusion. Infusions are made by tracking this meniscus and ejecting 0.05 μ l every 30 secs.
- 6.7.** When loading virus, use the air/water meniscus to measure loaded volume. Slightly overload the syringe then eject the excess against the sidewall of the storage tube. Again, monitor the ejected virus to ensure it is ejecting smoothly and with no bubbles. Leave a small excess of virus loaded in the syringe to ensure that the entire infused volume can be delivered and no air can enter the brain.
- 6.8.** Lower loaded syringes to the final D/V coordinates. Before infusing, raise arm up 0.1 mm. This creates a small pocket into which the vector can be infused and minimizes unintended diffusion of the vector up the track created by the injector. Begin the infusion by turning and applying gentle pressing to the plunger while monitoring the movement of the air/water interface. Manually infuse 0.05 μ l every 30

secs until the full volume is infused. Leave syringe in place for 7– 10 mins to avoid back-filling the needle track.

7. Complete surgery
 - 7.1. Remove syringes from the mouse brain. Remove the mouse from the stereotax.
 - 7.2. Use dissolvable sutures to close scalp incision. Alternatively, #7 wound clips may be used by collecting both sides of the incision with a sterile blunt forcep, pulling scalp to bring together in a small peak and securing with wound clips and Vetbond (if desired).
 - 7.3. Swab the incision with antibiotic ointment, refresh ophthalmic ointment, and administer 0.5– 1 ml of pre-warmed sterile saline subcutaneously and analgesic drugs as needed.
 - 7.4. Allow the mouse to recover in a clean, paper towel-lined cage on a heating pad. Monitor the mouse's breathing and temperature.
 - 7.5. When fully mobile, the mouse can be returned to a clean housing cage with fresh bedding. Consult your institution's animal care facility regarding special housing requirements. If wound clips are used in lieu of sutures, they can be removed after 5– 7 days.
 - 7.6. Clean needles between each surgery by ejecting remaining volume into 10% bleach to destroy remaining virus. Rinse syringe using 70% ethanol. Make sure that the volume enters the syringe without introducing bubbles. Repeat 70% ethanol rinse several times until syringes and needles are clean and free of bubbles. Follow with ultra-pure water rinse. Again, monitor to ensure that water is taken up cleanly without introducing bubbles. Prepare cleaned needle and syringe as previously described. If needed, replace needles between surgeries, and prepare as previously described.
 - 7.7. After surgery, needles can be cleaned using a fine-gauge cleaning wire.

BASIC PROTOCOL 3

Validation of virus expression *in vivo* using IHC

Stereotaxic delivery of viral vectors allows for a predictable time course of exogenous gene expression in targeted regions of the mouse brain. It is important to confirm that viral transduction is limited to the desired region, restricted to the appropriate cell type, and expressed at equivalent levels. Our labs favor IHC techniques to confirm placement and expression of our viruses. An alternative protocol for verification with western blotting is also included. Other techniques can be used for confirmation and include *in situ* hybridization to validate recombination or overexpression as well as placement, flow assisted cytometry sorting to prepare samples for quantitative polymerase chain reaction (qPCR) or western blotting, and laser capture microdissection for isolation of DNA or

mRNA from virally-transduced brain regions. Protocols detailing these techniques are widely available and should be consulted prior to use.

Materials—Fixed brains, prepared either by 3% PFA perfusion or drop-fixation, cryopreserved and sliced into 30– 50- μ m sections, stored in 1xPBS/0.01% sodium azide

NOTE: We find that drop-fixing mouse brains in fresh 3% PFA overnight is sufficient to preserve antibody detectable EGFP signals in AAV-transduced nucleus accumbens. Fixation conditions should be optimized for specific antibodies and viruses to ensure maximal detection sensitivity.

1X PBS

24-well plates

35-mm petri dish

Flame polished glass pipette, fashioned into a “hook” end

Trimmed paintbrush

SuperFrost+ Slides (Fisher, 12-550-15)

Microscope Cover Glass (coverslips) (Fisher, 12-545-M)

Slide Containers

Slide Racks

Ethanol Solutions (70%, 95%, 100% w/v prepared in ultra-pure water)

Citrosolve

DPX mounting media (HAZARDOUS)

Plastic Transfer Pipette

Pencil

IHC Blocking solution (see reagents and solutions)

Primary Antibody Solution diluted to appropriate concentration in Blocking Solution

Secondary Antibody Solution diluted to appropriate concentration in Blocking Solution

NOTE: If reclaiming antibody solutions, include 0.02% (w/v) sodium azide to prevent bacterial growth.

Rotary shaker or rotating platform

Counter staining solution (DAPI or Hoescht)

Placement Tracking Sheet with images of coronal sections of the brain region of interest for tracking targeting

Primary Antibody Exposure

1. Rinse slices from region of interest in 1X PBS.
 - 1.1. Using glass hook, transfer slices from storage plate into 35-mm petri dish filled with 1X PBS.
 - 1.2. Using glass hook, select slices from the region of interest and transfer to labeled 24-well plate filled with 0.5– 1 ml of 1X PBS.
 - 1.3. *NOTE: A photograph containing glass hook, 24-well plate, PBS, and petri dish for steps 1.1– 1.3 is provided in Figure 2A.*
 - 1.4. Wash slices 3 times, 5 mins per wash, by moving through wells.
2. Block slices for 1 hr in 1 ml of freshly prepared blocking solution.
 - 2.1. Shake gently at room temperature, protected from light.
3. Expose slices to primary antibody.

- 3.1. Transfer slices to well containing appropriate dilution of primary antibody (0.75– 1 ml/well).

The goal is to use a combination of primary antibodies that confirm the region of transduction. This can be accomplished by staining for a virally expressed fluorophore or for overexpressed proteins or protein tags. The goal is to maximize your ability to detect transduced regions in order to determine placement and exclude mice with failed or off-target expression. Initially, it is important to use TUNEL (or other cell death marker) as a counter stain. Although some cell damage is expected in the needle track and infusion region, significant overlap of apoptosis signal with transduction marker indicates that viral vector-mediated transduction is producing toxicity. Given the lack of toxic viral genes in most AAVs and HSVs, apoptosis could be a result of overexpression of the gene of interest or very high levels of fluorophore expression. Consider further in vitro experiments to assess toxicity of construct and altering infusion volume or duration of infection to reduce fluorophore build-up.

- 3.2. Determine appropriate exposure time, often overnight at 4°C.
 - 3.3. Shake gently at 4°C (if overnight exposure) or room temperature, protected from light.

If additional primary antibodies will be used, wash slices 3 times, 5 mins per wash in 1x PBS between exposures.

Secondary Antibody Exposure

4. Wash slices 3 times in 1X PBS, 5 mins per wash by moving through wells.
5. Expose slices to secondary antibody.
 - 5.1. Transfer slices to well containing 400– 500 µl of appropriate dilution of secondary antibody.
 - 5.2. Shake samples for 1 hr at room temperature, protected from light.

If additional secondary antibodies will be used, wash slices 3 times, 5 mins per wash in 1xPBS between exposures.

Counterstaining (optional)

6. Prepare DAPI or Hoescht stain (1:5000, in 1X PBS).
7. Incubate slices in 0.5 ml counterstain for 20 min at room temperature, protected from light.

Mounting—*NOTE: A photograph containing materials for mounting is provided in Figure 2A, B.*

8. Label SuperFrost+ slides using pencil.

Pencil must be used to clearly label slides as other writing materials will be removed or damaged by dehydration with ethanol and citrosolve solutions.
9. Transfer slices to 35-mm dish filled with 1X PBS using glass hook.
10. Using a fine bristled trimmed paintbrush, transfer slices to the slide oriented from anterior to posterior. Refer to a brain atlas for this step.
 - 10.1. Place paintbrush in 1X PBS near desired slice. Use gentle rolling motion to pick up slice and drape over paintbrush. Transfer paintbrush to slide and use gentle rolling motion to deposit slice on the slide.
 - 10.2. Rewet paintbrush as needed to help move slice. Use liquid to help orient slice without tugging or tearing.
 - 10.3. Remove excess liquid from around slice with kimwipe prior to mounting next slice. Avoid excessively wetting the slide, as it will cause slices to float from desired orientation.

Alternate technique: Submerge slide completely and use paintbrush to load slices onto slide. Remove slide from PBS once all slices on glass and use paintbrush to correct final orientation.
 - 10.4. Leave slides to dry, protected from light, for a minimum of 30 mins

Slides can be left to dry overnight if needed.

Dehydration and Coverslipping

11. All washes and coverslipping should take place in a fume hood.

12. Prepare slide containers with the following solutions:
 - 12.1. 70% (w/v) Ethanol
 - 12.2. 95% (w/v) Ethanol
 - 12.3. 100% (w/v) Ethanol
 - 12.4. 3 containers with Citro-solve
13. Load a small number (6 or less) slides into plastic rack with label to the top.

Once dehydrated, it is important to complete mounting steps quickly. Limit the number of slides processed at any given time to avoid drying out or damaging the slices.
14. Perform a series of washes by transferring the slide rack from each container using the following schedule:
 - 14.1. 70% Ethanol for 20 secs
 - 14.2. 95% Ethanol for 20 secs
 - 14.3. 100% Ethanol for 20 secs
 - 14.4. Citro-solve for 20 secs
 - 14.5. Citro-solve for 1 min
 - 14.6. Citro-solve for 5 mins
 - 14.7. Keep entire wash time to less than 10 mins.
15. Remove slide rack from last wash container and place on paper towel.
16. Remove one slide at a time and wick excess Citro-solve off on paper towel

It is critical to avoid over-drying slides. Ensure that all slides are coverslipped within 2 mins of removing from the last wash step. It is best to process a small number of slides at a time to ensure that dehydration, washing, and coverslipping takes less than 12 mins total.
17. Using a plastic transfer pipette, add 3– 4 drops of DPX mounting medium across the slide surface.
18. Quickly place coverslip on one edge of slide imaging area, and lower slowly but steadily. Avoid introducing bubbles if possible.
19. Store slides in a covered container and allow to dry overnight.

Imaging

20. Consult mouse brain atlas to determine targeted region.
21. Image slides an appropriately outfitted microscope.
22. Confirm expression using fluorescence signal; confirm targeting by comparing to atlas images.

23. Log targeting, extent of expression, and general health of tissue.
24. Take representative images of spread for all viruses and groups. Virus spread can be different between constructs but must be matched for inclusion in experiment.
25. Update coordinates as needed to improve targeting.

ALTERNATE PROTOCOL 1

Validation of virus expression *in vivo* using western blotting technique

In addition to confirming expression in targeted brain regions, western blotting techniques can be used to compare the change in expression of proteins of interest following viral-mediated gene expression. This alternate protocol describes general techniques for acquiring and preparing tissue from infected brain tissue for western blotting analysis and quantifying protein using the LiCor Odyssey two-color detection system.

Materials—Mice with virally transduced brain region

Guillotine or approved sharp decapitation scissors

Dissection equipment

Blunt forceps, fine forceps, small scissors, rongeurs, punches, razor blades, and brain matrix. 1-mm or 0.5-mm matrices are available in both sagittal and coronal orientations (*e.g.*, Alto 1-mm mouse coronal matrix, Roboz).

NOTE: Tissue punches can be purchased (*e.g.*, NIH style neuropunch, Fine Science Tools) or constructed by removing the beveled edge of different gauged needles using a small dremel saw.

Dry ice

Wet ice

Large weigh boats or 35-mm petri dishes

Aluminum Foil

1-ml syringes

28-G needles

1X PBS

1.7-ml micro centrifuge tubes (labeled)

Sucrose Lysis Buffer (see reagents and solutions)

Protease, Kinase, and Phosphatase inhibitors as needed

Sample product: Mini Protease Inhibitor Tablets (Roche)

NaF (50 mM), Okadaic Acid (0.5– 50 nM), Sodium Orthovanadate (1 mM), pMSF (1 mM)

Sonicator (wand-style, *e.g.*, Cole-Parmer Ultrasonic Processor)

MilliQ or other ultra pure water

70% ethanol (w/v)

Water bath, heat block, or other unit capable of boiling tubes

Tabletop centrifuge

BCA or other protein assay kit

Protein Sample Buffer (SAB)

Reducing Agent, *e.g.*, β -mercaptoethanol (β -ME) or dithiothreitol (DTT)

SDS-PAGE electrophoresis system

Western blotting transfer system

LiCor Odyssey Infra-Red Imaging System

1X PBS

1X TBS-T

10% (w/v) Tween-20

10% (w/v) SDS

Optional for dissection:

Additional light sources

Dissecting microscope

NOTE: All protocols using live animals should be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC). Trained personnel, following procedures approved by IACUC and other institutional regulations pertaining to animal welfare, should conduct all dissections.

Preparation of Workspace

1. Prepare one large, rectangular ice bucket filled with wet ice and cover workspace with aluminum foil.
2. Prepare one sufficiently sized ice bucket with dry ice. Be sure there is space for all samples to be fully inserted into dry ice.

3. Label all tubes with mouse and brain region identifiers, as well as with the date.
4. Fill one weigh boat or petri dish with iced 1X PBS. This dish will be used to rinse and cool brains prior to insertion into the brain matrix.
5. Fill two weigh boats with 1X PBS. Store these boats on the aluminum foil work surface of the ice bucket. Place 1-mm brain matrix, matrix side down, submerged in the PBS of one boat. The other boat will be used to collect the slices generated from the matrix and collect tissue.
6. Unwrap razor blades, assemble punches onto 1-ml slip tip syringes, and array all dissecting equipment within easy reach.

Rapid Decapitation and Tissue Collection

7. Rapidly decapitate mouse according to IACUC regulations for your institution.
8. Rapidly remove whole brain.
 - 8.1. Cut scalp to expose the skull.
 - 8.2. Flip head upside down and make three small cuts at the skull around the brain stem. The goal of these cuts is to break the skull on dorsal surface without damaging the brain. One cut should be made on either side medial/lateral of the brain stem and the last cut should be made at the posterior aspect. This cut should crack the skull ideally as far as the interparietal suture.
 - 8.3. Flip head right-side up and locate crack in interparietal bone produced by the cut. Using blunt forcep, pull up on parietal bone at sagittal suture to remove skull. Remove by pulling straight up; avoid twisting movements that can drive bone fragments into the brain tissue.
 - 8.4. Expose brain by removing skull as far anterior as possible. Using flat spatula, run around the outer edge of the brain to clear away any bone and pull meninges away. Using flat spatula, run under the brain to lift away and scoop out of skull.
9. Place brain into the weigh boat containing iced 1X PBS and let rest for 1 min.
10. Load chilled brain into the brain matrix.
11. Anchor brain in matrix by placing one razor blade at the anterior aspect of the brain and gently pushing forward with a flat spatula to fully seat the brain in the matrix. Place a second razor blade at the cerebellum to anchor.
12. Place razor blades every 1 mm through the region of interest.
13. Remove razor blades, gently and one at a time. Tissue slice will be resting on the blade, float tissue off into weight boat containing 1X PBS.
14. Identify slices that contain region of interest and subdissect tissue of interest.

- 14.1.** Use fine forceps to remove larger regions like hippocampal formation or dorsal striatum.
- 14.2.** Use a tissue punch to remove smaller regions like nucleus accumbens or cortical subregions.

- 14.2.1.** Fit punch on 1-ml slip tip syringe. Prime syringe by pulling up a small volume of 1X PBS.

- 14.2.2.** Place punch over region of interest and push down. Twist punch while gently drawing up to free region of interest and draw through punch into syringe.

Avoid pressing with too much force. It is easy to puncture thin walled weigh boats, causing drainage of PBS. For difficult regions, consider using a forceps to separate punch from surrounding tissue before drawing up.

- 15.** Place tissue into appropriate labeled tube and remove all excess solution with a fine gauge syringe.

Remove as much PBS as possible prior to flash freezing on dry ice. Remaining liquid will damage cell walls and cause deterioration of tissue sample.

- 16.** Place tissue-containing tubes into dry ice; make sure entire tissue is submerged into dry ice.

- 17.** Collect pertinent control regions as necessary.

- 18.** Tissue can be stored at -80°C until ready to prepare lysate.

Tissue collected in this manner can be processed for protein lysate as described below OR processed to collect mRNA.

NOTE: This protocol is for immediate tissue collection after euthanasia. Alternatively, intact brains can be snap frozen using dry ice and ice-cold isopentane after decapitation, stored at -80°C , and sectioned later on a cryostat. When this method is used, tissue samples are collected at the time of sectioning. When taking samples in a cryostat, ensure that tissue punches are completely dry. Any moisture inside will rapidly freeze and occlude the tissue punch. Samples can be stored at -80°C or immediately placed into lysis buffer (see Preparation of Protein Lysate below).

Preparation of Protein Lysate

- 19.** Prepare complete sucrose lysis buffer by adding inhibitors to appropriate working concentration.

- 20.** Add 50– 200 μl of lysis buffer to each sample.

Volume added is dependent on size of sample – for example, 50 μl for nucleus accumbens punch, 150 μl for whole hippocampus, 100 μl for dorsal striatum.

Ideally, the volume should cover the tissue and provide sufficient space to sonicate completely.

21. Sonicate samples with wand at low amplitude (15– 50%). Avoid making contact with the side of the tube. Sonicate for 10 sec per sample; avoid over heating the sample. Samples can be sonicated for additional time after resting on ice.
22. Store sonicated samples on ice.
23. Clean wand using ultra-pure water, 70% ethanol, and ultra-pure water. Clean between all samples.
24. Boil sonicated samples for 10 min in a 98°C water bath.
25. Cool samples on ice for 5 min.
26. Spin samples at 16,000 ×g for 10 min at 4°C.
27. Prepare new tubes for all samples, and store on ice.
28. Transfer supernatant to pre-chilled tubes; be careful to avoid disturbing the pellet.
29. Take sample for protein assay, store remainder of lysate at –80°C until concentrations are determined.

Alternatively, samples can be stored on ice while concentration is determined if this can be completed in a short time period.

30. Adjust concentrations of all samples to equal amount using lysis buffer and add 4X SAB (complete with fresh β -ME or DTT).
31. Boil SAB containing samples at 98°C for 10 min, spin 10 min, and store at –20°C.

Electrophoresis and Visualization of Protein

32. Determine appropriate SDS-PAGE concentration for optimal separation of proteins of interest.

Consult alternative protocols for more detailed discussion of Western Blotting technique and considerations. Run and transfer all gels for a given experiment using the same settings (gel concentration, running and transfer conditions, antibody exposure times) to reduce variability.

33. Load equal quantities of tissue lysate by normalizing to total protein concentrations for all conditions on the same gel, when possible.

Alternate between experimental and control samples when loading sample into lanes.

34. Run gel at an appropriate voltage for sufficient time to maximize separation of bands of interest, both experimental and loading control.
35. Transfer proteins to membrane for antibody exposure.

Consider staining gels with coomassie blue or membranes with Ponceau after transfer to ensure complete transfer and adjust transfer protocol as needed to optimize efficiency.

36. Block membrane for 1 hr at room temperature with gentle shaking. Use 30 ml of LiCor Blocking Buffer diluted 1:1 in 1X PBS.
37. Wash membrane 3X in 1X TBS-T, 5 min per wash with gentle shaking.
38. Expose membrane to 30 ml of primary antibody solution diluted to appropriate concentration in blocking buffer with 0.1% Tween-20 added. Determine exposure time (see comment) and keep covered and shaking gently during exposure period.

For high abundance proteins often used as loading controls (e.g., β -tubulin), exposure times can be as short as 30 min at room temperature. For most experimental proteins standard exposure is 1 hr at room temperature. For low abundance proteins, consider an overnight (~16 hr) incubation at 4°C with gentle shaking.

Volumes can be adjusted to the size of the membrane being probed. Ensure that all portions of the membrane are completely submerged, even while shaking.

In addition to probing with a loading control, if possible also include a control for viral infection efficiency. This is best accomplished by blotting for a fluorescent protein (e.g., EGFP) if included in the viral vector used.

39. Reclaim primary antibody solution and store at 4°C.
40. Wash membrane 3X in 1X TBS-T 5 min per wash with gentle shaking.
41. Complete additional primary exposure as needed for two-color detection system. Follow with wash step prior to secondary exposure.
42. Expose membrane to 30 ml of secondary solution diluted to appropriate concentration in blocking buffer, and include 0.1% (v/v) Tween-20 and 0.01% (w/v) SDS for the secondary step.

It is recommended when using a two-color detection system to put your most abundant protein (loading control) in the 700 nm (red) channel.

43. Expose membrane to secondary solution for 1 hr at room temperature with gentle shaking. Make sure membrane is protected from light.
44. Wash membrane 3X in 1X TBS-T 5 min per wash with gentle shaking.
45. Image membrane according the manufacturer's specifications.
46. Scan your blot with protein side down, wet or dry, directly on imaging surface.

Quantification and Analysis of Protein

47. Normalize band intensity values to the loading control from the same lane.

48. Consider normalizing again to the transduction efficiency marker if available and appropriate.
49. Compare normalized values using t-tests or ANOVAs as appropriate.
50. Consider follow-up examinations of remaining lysate to examine changes in related proteins or expected downstream changes due to expression of your construct of interest.

SUPPORTING PROTOCOL 1

Determination of appropriate virus infusion volume and time course of expression

Variability in the transduction efficiency of AAV vectors is one consequence of the increasing number of serotype/capsid combinations available. While HSVs exhibit robust neurotropism, other factors, like helper virus concentration and promoter type can impinge on transduction efficiency of these vectors. Thus, for both AAV and HSV vectors, it is critical to determine the appropriate infusion volume and expression pattern of constructs prior to starting virally-mediated gene transfer experiments. This support protocol provides a simple framework for designing pilot experiments to optimize the use of either vector type *in vivo*.

Materials

Aliquoted high-titer viral vector (see Basic Protocol 1)

Stereotaxic equipment and animals (see Basic Protocol 2)

Materials for IHC validation of virus expression (see Basic Protocol 3)

Determining optimal infusion volume and time course of expression

1. Infuse a range of volumes. Start with 0.1 μ l increments from 0.3–1.0 μ l (recommended for AAV) and 0.5– 2.0 μ l (recommended for HSV), depending on desired region. Multiple independent infusions sites are preferable to large injection volumes when targeting a large structure (*e.g.*, dorsal striatum) or multiple sub-regions within the same structure (*e.g.*, hippocampal subdivisions).
2. Different infusions volumes can be administered to each side of a single mouse in order to limit the number of mice required for pilot experiments.
3. Generate sufficient mice to examine expression at multiple time points. Most AAVs will have maximal infection by 2– 3 wks after infection, while HSVs are associated with peak infection much earlier, at approximately 3– 5 days post injection. Depending on the experimental design, these specific time courses can be considered either a benefit or a limitation of the vectors. This is discussed in more detail in the Commentary section (Background and Timing of expression and experimental design).
4. Confirm peak expression and demonstrate maintained expression at time points beyond preferred experimental timelines. Use IHC protocol to determine extent of spread and tissue damage with infused volume.

5. Smaller, well-targeted infusion volumes are preferable to volumes that fill the region of interest but produce off-target expression as well. Ideal on:off target ratios should be well above 75:25% of signal.

REAGENTS AND SOLUTIONS

IHC Blocking Solution

3% (w/v) Bovine Serum Albumin

0.3% (v/v) Triton-X100

0.2% (v/v) Tween20

3% (v/v) normal donkey/goat serum

NOTE: Match serum to species that secondary antibody was raised in

In 1X PBS

Sucrose Lysis Buffer

5mM HEPES (pH 7.4)

1% (w/v) SDS

0.32M Sucrose

in Water

Protein Sample Buffer (NOTE: Displayed as 1X, Prepared as 4X)

50 mM Tris-HCl pH 6.8

2% (w/v) SDS

10% (v/v) glycerol

1% (v/v) β -ME

12.5 mM EDTA

0.02% (v/v) bromophenol blue

LiCor Solutions

Blocking: LiCor Blocking Solution (LiCor, 927–40000) and 1X PBS in 1:1 dilution

Primary Antibody: 1:1 Diluted LiCor Solution and 0.1% Tween-20

Secondary Antibody: 1:1 Diluted LiCor Solution, 0.1% Tween-20, and 0.01%

SDS

COMMENTARY

Background Information – Adeno-associated Viruses

Recombinant adeno-associated viruses (AAVs) have several properties that make them ideal for use in the adult brain. AAVs are nonpathogenic parvoviruses which are naturally

replication deficient in the absence of helpervirus co-infection (Berns and Giraud, 1996; Xiao et al., 1997). Infection by AAVs produces limited immune responses and the AAV backbone contains essentially no toxic viral genes (Kaplitt et al., 1994). AAVs are capable of infecting broad cell types, including both dividing and non-dividing cells (Flotte et al., 1994; Podsakoff et al., 1994). Once a cell is infected, the AAV genome is retained in cells as episomal concatemers (Schnepp et al., 2003), a configuration that maintains expression of the viral genome for long periods of time while limiting genomic integration. The transduction efficiency of AAVs depends, in part, on the particular type of capsid proteins that regulate the selection of cell-surface receptors that are engaged for import into the cell. AAV subtypes utilize distinct cap proteins, producing differing affinities for specific cell types. In recent years, an increasing number of AAV sub-types have been identified, increasing the combination of “pseudotyped” (referring to the specific combination of inverted terminal repeats [ITRs] and capsid proteins from different serotypes) AAVs available for experiments (Xu et al., 2005; Cearley et al., 2008; Liu et al., 2008). These pseudotypes have different cellular tropisms, and recent studies have identified preferred pseudotypes for different mouse brain regions and cell types (Davidson et al., 2000; Rabinowitz et al., 2002; Wang et al., 2003; Cearley and Wolfe, 2006; Taymans et al., 2007; Aschauer et al., 2013; Watakabe et al., 2014a).

AAVs are an appropriate vector for a variety of applications including over-expression or recombination, sh/siRNA-mediated knockdown (RNAi), and tract tracing. The primary limitation in the use of AAVs is their limited construct size. Due to the AAVs small protein capsule, inserted constructs are limited to 4.5–5.0 kb (Dong et al., 1996). Although this limitation can interfere with expression of large proteins, there are numerous strategies developed to deal with this problem (see Critical Parameters section).

The AAV platform is often coupled with the Tet-ON or Tet-OFF system where a single vector contains the transgene of interest under the control of the tet-O sensitive promoter element and a second constitutive promoter driving expression of the transactivator protein (Le Guiner et al., 2014; Watakabe et al., 2014b). Recently AAVs have been successfully used for targeted gene editing using the CRISPR/Cas9 system (Swiech et al., 2015) and neuronal activity manipulation using optogenetic constructs (Gradinaru et al., 2010; Yizhar et al., 2011). Tract-tracing and connectivity studies often use AAVs with DIO or FLEX sequences that allow injection of the AAV into brain regions with transgene expression under control of Cre recombinase activity (Wouterlood et al., 2014) or take advantage of different AAV serotypes that demonstrate robust antero- or retrograde transport with varying degrees of 2nd order transduction to define connected brain regions (Salegio et al., 2012; Rothermel et al., 2013; Castle et al., 2014; Wang et al., 2014).

With proper considerations for experimental goals (see Critical Parameters and Troubleshooting) AAVs are a reliable method for long-term *in vivo* expression of transgenes. The AAV platform has a wide variety of applications as evidenced by their growing use in neurobiology research.

Background Information – Herpes Simplex Virus

Many of the earliest studies in which viral-mediated gene transfer was used to establish causal relationships between genes and behavior involved HSV vectors. HSVs are neurotropic viruses; as such, they can robustly infect both dividing and quiescent neurons *in vitro* and *in vivo* (Lim, 2013). This property of HSVs negates the need for sero- or pseudotyping to increase neuronal affinity (Lim, 2013) and lends itself to gene transfer in species that are less permissive to other forms of virally-mediated gene transfer, like the zebrafish (Zhu et al., 2009; Zou et al., 2014).

HSV vectors have low cytotoxicity and pose low risk for insertional mutagenesis, as their viral genome is not readily incorporated into the host's chromosomes (Neve et al., 2005; Jerusalinsky and Epstein, 2006; Jerusalinsky et al., 2012; Lim, 2013). There are three classes of HSV vectors for transgene delivery: replication-attenuated, replication-deficient, and amplicon vectors (Frenkel, 2006; Marconi et al., 2015). Replication-attenuated and replication-deficient genomic HSV vectors have the transgene of interest inserted into the viral genome, with targeted deletion of specific immediate early (IE) genes to disable the lytic cycle and render these vectors non-toxic (Wu et al., 1996; Krisky et al., 1998). These vector subtypes have been discussed in detail elsewhere (Fink et al., 1996; Simonato et al., 2000; Mata et al., 2002; Goins et al., 2004). The focus of the present commentary is on amplicon vectors: “empty” plasmids containing little to no native viral DNA (Neve et al., 2005). Amplicons are arguably the most malleable HSV vectors due to their small plasmid size (5–10 kb), which permits efficient cloning (Neve and Lim, 2013).

Historically, a limitation of HSV amplicons has been the requirement for helper-viruses, as virtually no cell lines have been made that express all essential proteins for packaging into HSV particles (Spaete and Frenkel, 1982; 1985; Neve et al., 2005; Cuchet et al., 2007). Even replication-deficient helper viruses have been shown to produce some cytotoxicity (Neve and Lim, 2013), and studies suggest that the greater the number of IE deletions to the helper virus, the lower the expected titer (Lim et al., 1996). However, progress in vector development has lead to high-titer amplicons with improved amplicon/helper virus ratios (Neve and Lim, 2013), CRE-dependent helper viruses yielding nontoxic amplicons (Logvinoff and Epstein, 2000; Zaupa et al., 2003), and helper virus-free systems using bacterial artificial chromosomes to provide the HSV genome (Stavropoulos and Strathdee, 1998; Saeki et al., 1998; Horsburgh et al., 1999; Saeki et al., 2001).

Similar to AAVs, the potential applications for HSV vector systems are broad and include gene overexpression (Carlezon et al., 1997; Carlezon et al., 1998; Kelz et al., 1999; Carlezon et al., 2000; Pliakas et al., 2001; Rumpel et al., 2005; Gräff et al., 2014; Scobie et al., 2014), mutations resulting in loss of function (Han et al., 2008; 2007; Muschamp et al., 2011; Loweth et al., 2013), and siRNA-induced gene knockdown (Hong et al., 2006; Yang et al., 2011). Moreover, HSVs are particularly well suited for circuit tracing, in that most vector subtypes are subject to retrograde transport (Norgren and Lehman, 1998). Several recent reports have demonstrated that use of retrogradely-transported HSVs expressing Cre recombinase, combined with local injections of Cre-dependent AAVs encoding channel- or

halorhodopsin-2, enables projection-specific optogenetic control of neural circuits (Jennings et al., 2013; Stamatakis et al., 2013; Senn et al., 2014; Fenno et al., 2014).

HSVs have been effectively utilized to deliver optogenetic and DREADD constructs (Covington et al., 2010; Christoffel et al., 2011; Ferguson et al., 2011; Michaelides et al., 2013), and are particularly well suited for delivering large constructs, owing to their outstanding transgene capacity – up to 160 kB (Saeki et al., 2001; Jerusalinsky and Epstein, 2006; Cuchet et al., 2007). Thus, HSV amplicons can accommodate large or multiple transgenes along with their essential regulatory sequences, improving the physiological relevance of gene transfer manipulations (Lim, 2013). Recently, the HSV system has been elegantly coupled with Brainbow technology for circuit tracing (Zhang et al., 2015). Whereas AAV-Brainbow constructs encode 1–4 hues (Cai et al., 2013), HSV-brainbow vectors were demonstrated to express between 120–165 hues, labeling individual neurons with unprecedented resolution (Zhang et al., 2015).

The majority of HSV vectors, particularly “first generation” ones, have rapid and transient infection profiles. As such, they lend themselves nicely to experiments involving repeated testing - “recovery” experiments - in which behavioral adaptations tightly correspond with rising and falling transgene expression, enabling elegant demonstrations of cause-effect relationships. They are not an ideal platform for studies requiring sustained, if not permanent, alterations in gene expression, which are better suited for AAV use (see Timing of expression and experimental design). When used properly, both vector types can provide invaluable and complementary insights into brain function. These protocols are meant to serve as guidance for researchers wishing to introduce AAVs or HSVs into the adult mouse brain and provide multiple methods for validating the extent of infection and transgene expression.

Critical Parameters and Troubleshooting

Construct design—The most critical parameter for *in vivo* viral vector use is the design of the construct itself. Although these protocols are focused on the use of a prepared vector, this section includes a discussion of key vector design considerations. HSVs do not require pseudotyping to increase neurotropism, as these viruses are inherently neurotropic (Neve et al., 2005). AAVs, on the other hand, benefit from pseudotyping. As the number of distinct AAV subtypes identified increases, the options of available pseudotyped AAVs also increase. Proper selection of the capsid proteins used will affect the relative tropism for particular cell types and viral spread in particular brain regions. Some serotypes exhibit anterograde and/or retrograde transport (*e.g.*, AAV1, AAV8, AAV9), making them suitable for tract-tracing but potentially complicating experiments that require tight control of regional expression. Findings from previous publications investigating the use of specific AAV serotypes in the mouse brain have been extensively reviewed elsewhere (Davidson et al., 2000; Rabinowitz et al., 2002; Wang et al., 2003; Cearley and Wolfe, 2006; Taymans et al., 2007; Aschauer et al., 2013; Watakabe et al., 2014a).

Promoter selection greatly influences which cells express the transgene and to what extent it is expressed. Many AAV and HSV platforms use promoters with a high level of constitutive activity, particularly the CMV (cytomegalovirus) or related CAG (combination of the CMV

immediate early enhancer and the chicken β -actin promoter) and the IE4/5 and CMV promoters for AAV and HSV, respectively. Although these promoters produce high levels of transgene expression, they do so in a cell-type independent manner. If cell-type specificity is desired, the use of broad cell-type specific promoters to restrict expression to neurons or glia (*e.g.*, Synapsin-1, GFAP) or more restricted promoters for neuronal cell type-specific expression (*e.g.*, TH, Emx1) will greatly enhance the selectivity of both AAVs and HSVs. Notably, evidence suggests that use of neuronal, as opposed to viral, promoters in HSV vectors drives longer-lasting gene expression *in vivo* (Jin et al., 1996; Neve et al., 2005).

When using AAV and HSV platforms for expressing small, non-coding RNAs (*e.g.*, siRNA or shRNA) and/or expressing two distinct open reading frames, there are some special considerations. For applications where expression of an shRNA is desired, the use of a promoter that recruits RNA polymerase III enzymes (*e.g.*, H1 or U6) is commonly used. When designing constructs with two promoters, the use of two distinct promoters can reduce promoter competition for transcriptional machinery (*e.g.*, cell-type specific for transgene, ubiquitous promoter for fluorophore), but reductions in gene expression from both open-reading frames should still be expected.

As noted previously, one significant limitation of AAVs is the small size available for transgene insertion (~4.5–5 kB between the ITRs). Larger constructs containing cell-type specific promoters and expressing multiple transgenes along with their regulatory elements are better suited for HSV vectors, which can accommodate ~160 kB. However, many groups have successfully exploited the available space in AAV to express both the transgene of interest and an unfused fluorophore, using either an internal ribosomal entry sequence (IRES) (Atasoy et al., 2008; Gradinaru et al., 2010) or two-promoter constructs 1. Other techniques to deal with AAV size limitation include reducing the size of the expression cassette (Husain et al., 2009; Choi et al., 2014) or co-delivery of multiple AAVs, each expressing a portion of the transgene of interest (Ghosh et al., 2011; Palfi et al., 2012; Swiech et al., 2015).

Timing of expression and experimental design

Careful consideration of experimental design and desired timeline of expression is essential when selecting a viral vector. The time course of viral expression is one of the principal differences between AAVs and HSVs. AAV-mediated gene expression has a slow onset (~7–10 days with peak expression at 2–3 weeks) and persists for months to years. On the other hand, HSV-mediated gene expression has a rapid onset (~12 h) but typically dissipates after ~8–10 days. Thus, HSV vectors are ideal for “recovery” experiments, where loss or gain of function is achieved via viral vector-mediated gene expression, but the effect is transient, allowing for the testing of a biological effect before, during, and after expression of a protein of interest. However, it is important to note that these expression parameters may vary among different batches of virus, and it is essential to rigorously validate expression parameters prior to selecting time points for repeated behavioral analysis. For these analyses, consider techniques with sensitivity to detect expression at lower levels (*e.g.*, qPCR, *in situ* analysis), which will benefit detection of pre or post-peak virus expression.

Unlike HSVs, AAVs are more suitable when enduring gene expression manipulations are desired. Due to their lag in maximal expression and the robust recovery of adult mice from the described surgical procedures, AAVs offer a unique advantage over HSVs in that it is possible to obtain baseline measures from subjects after surgery but prior to transgene expression. Some investigators have overcome this apparent limitation of HSV by pre-implanting guide cannulas into target regions, which enables collecting baseline data after surgery, delivery of the vectors to awake or lightly anesthetized animals, rapid analysis of the consequences of altered gene expression, and confirmation of recovery corresponding to loss of transgene expression (Todtenkopf et al., 2006; Muschamp et al., 2011). Like experimental design using AAVs, it is critical to determine expression parameters in order to capture a time point minimally influenced by surgery stress but prior to the onset of viral expression.

It should be noted that use of HSVs and AAVs is not limited to short and long experimental designs, respectively. For instance, incorporation of Tet-ON or Tet-OFF constructs into AAV vectors can provide temporal control of viral vector-mediated gene expression. With proper validation of transgene activation, subjects can be examined repeatedly following induction or repression of expression. In parallel, several strategies have been offered for conferring prolonged HSV-mediated gene expression, including use of a neuronal, rather than a viral promoter (Jin et al., 1996; Neve et al., 2005) and construction of hybrid HSV-AAV (Fraefel et al., 1996; Johnston et al., 1997; Costantini et al., 1999). Furthermore, there is a new line of commercially available long-term HSV vectors that are stably expressed for up to 28 days *in vivo* (Jennings et al., 2013; Stamatakis et al., 2013; Yonehara et al., 2013). They are subject to robust retrograde transport, however, and may not be ideal for experiments designed to maintain gene expression regionally for an extended duration.

Infusion volume

The optimal infusion volume of virus is highly dependent on virus type (*e.g.*, AAV vs HSV), titer, brain region, promoter, serotype/pseudotype, and helper virus, if applicable. Thus, despite the availability of a large database of infusions volumes into various brain regions in the literature, it is extremely important to experimentally determine the necessary infusion volume each time a new virus and/or brain region is investigated.

An infusion volume of 0.5–1 μ l is recommended for expression of AAV into most brain regions, though volumes in the tens of nanoliters have been reported. A substantially larger infusion volume is often required for HSV vectors. In unpublished work from our lab, we evaluated transgene expression in the VTA following infusion volumes of HSV ranging from 1–2 μ l of a full-strength titer (10^8 IU/ml) and compared these results to the effects of 1.0 μ l or 2.0 μ l of a 50% diluted titer. Independent of titer, transgene expression *within* the VTA was greater following 2.0 μ l, relative to 1.0 μ l, microinjections of HSV. As expected, the higher-titer virus yielded better expression than the diluted titer virus. However, injection volume was a better predictor of expression than titer. These findings may indicate that elevated titer simply results in a larger (asymptotic) number of transductions per cell within the diffusion radius of the injection, which is determined by injection volume. This

parametric work illustrates the importance of in house validation of optimal infusion volumes prior to experimentation.

Control considerations

Considering the range of possible experiments that can use *in vivo* AAV- or HSV- mediated gene expression, proper controls will depend on the specific experiment. Over-expression of one or more genes of interest is the most common application of viral vectors. Often the most straightforward control for this type of experiment is a group of mice with an AAV- or HSV-mediated fluorescent protein expression (“empty vector”) in the same brain region. Although this design effectively controls for the non-specific consequences of surgery and microinfusions of a viral vector, alternatives exist. Constructs for over-expression often take advantage of two promoters to express the gene of interest and a fluorescent protein. Promoter competition *in vivo* may lead to reduced levels of expression of both parts compared to a single promoter driving a fluorophore. Experiments often include use of either null versions of the gene of interest or a similarly sized but non-functional protein (*e.g.*, LacZ, Luciferase). An equally popular use for AAVs in particular is Cre-mediated recombination of loxP-flanked DNA regions in a genetically-modified mouse. For these experiments, the “empty vector” control is the most commonly used control group, but it is also possible to express a mutated form of Cre recombinase, which lacks enzymatic activity and serves as a better control. Additionally, the inclusion of a group receiving AAV-Cre, but lacking the floxed allele, will enable the detection of non-recombination mediated Cre expression effects. As a positive control to identify cells where Cre-mediated recombination has likely occurred, some labs have used a loxP-reporter mouse that will express a reporter gene (*e.g.*, EYFP or tdTomato) following successful Cre-mediated removal of a floxed transcriptional stop cassette (Muzumdar et al., 2007). Another common application for both AAVs and HSVs is *in vivo* delivery of shRNA or siRNA (RNAi) constructs for mRNA knockdown. The primary consideration in these experiments is the need to validate that the primary mRNA target, and not off-target effects, mediate the phenotype of interest. There are a number of techniques that can be used to demonstrate target-specific effects, including expressing two or more shRNAs that target unique parts of the mRNA of interest to demonstrate the same phenotype or rescuing the phenotype by simultaneous expression of the RNAi and expression of an RNAi-resistant form of the targeted mRNA. In addition to these RNAi-specificity controls, it is also critical to have appropriate negative controls for the surgery and introduction of the virus. Rather than an “empty vector” control, negative controls for RNAi experiments often consist of either scrambled RNAi sequences or target sequences directed against non-genomic sequences (*e.g.*, LacZ or luciferase).

Viral vector-mediated gene transfer for *in vivo* studies has a high experimental attrition rate. Mice are often excluded due to improper targeting or expression, which increases the number of subjects that must be generated. This high attrition reinforces the importance of properly designing control experiments to include required groups and carefully examined pilot experiments for expression and placement in order to maximize the usefulness of each mouse generated.

Protocol specific troubleshooting

Basic Protocol 1 is a simple protocol for the subaliquoting and storage of commercially or core-prepared virus. Existing protocols are available to help researchers generate their own vectors. It is critically important that viruses be stored in low-binding tubes to allow maximal recovery of the volume, but more importantly, to avoid retention of viral particles by the tube itself. Using other tubes will cause reduced transduction efficiency due to what is essentially a reduction in viral titer.

Basic Protocol 2 is a simplified protocol describing stereotaxic delivery of viral vectors into the adult mouse brain. The primary problems that will be encountered during stereotaxic surgery relate to mouse anesthesia, either completeness or recovery. Although we describe ketamine/xylazine anesthesia, researchers may want to consider using inhaled anesthetics (*e.g.*, isoflurane) to better control the extent and duration of anesthesia. In addition, it is now widely appreciated that ketamine can produce persistent behavioral (antidepressant-like) effects, which may complicate the experimental design and data interpretation. To circumvent this issue, consider the use of alternate surgical anesthetics (*e.g.*, isoflurane, pentobarbital). When using ketamine/xylazine anesthesia, consider co-administering atropine (0.02–0.05 mg/kg, IP) to reduce the build-up of bronchial secretions that can cause breathing difficulties. Ketamine/xylazine anesthesia can be effectively terminated through administration of a reversal agent such as the α -2 adrenergic antagonist atipamezole (1.0 mg/kg, IP). It is critical that reversal agents only be used at the end of a surgical procedure because the duration of anesthesia will be significantly shortened. The most common problem encountered in stereotaxic surgery is a failure to accurately target the brain region of interest. Initial experiments with a handful of possible targets will help determine ideal coordinates but targeting is ultimately determined by proper placement of the mouse into the stereotaxic frame. Ensuring that the skull is “flattened” prior to targeting is a key step in ensuring calculated coordinates are accurately reached. When measuring D/V deviations from bregma, make sure the needle is at a 0° angle and take measurements in both M/L and A/P directions. Select measurement locations that exceed your targeting range (*e.g.*, if coordinates are 1.5 mm from bregma, measure for flatness at 2 mm from bregma). Remember that small deviations in D/V measurements proximal to bregma will be amplified at more distal locations. Attempt to flatten the mouse (by raising/lowering the nose piece or ear cup as needed) to within 0.1 mm between measurements. Sometimes it will be necessary to remount the mouse entirely to improve orientation. Plugging of the needle is a common problem in stereotaxic surgery. Using smaller gauge needle and thoroughly cleaning or replacing needles between mice can help reduce this problem, although adjustments should be made in the infusion rate when increasing the gauge (*i.e.*, reducing the bore diameter) of the injector.

Basic Protocol 3 is a simplified protocol for IHC analysis of virus expression and placement. Critical considerations in this experiment include the use of specific primary antibodies, optimizing exposure times for antibodies, and accurate accounting of targeting. This protocol works best with 30–50 μ m thick sections; variations in thickness can alter recommendations for this protocol. Thinner sections are very difficult to work with and can easily be damaged by transfers using glass hooks. For thinner sections, using net-wells to

hold and transfer slices during washes and antibody exposures can reduce the risk of damage. Net-wells placed in a 24-well plate will require additional volume to fully submerge sections. It is also advised to mount thinner slices by submerging the slide (as noted in protocol) instead of mounting with the paintbrush. When using thicker sections, antibody permeation through the tissue (as well as imaging clarity) can become limited. Increasing the duration of the permeabilization step and increasing the concentration of primary antibody can sometimes improve permeation. As section thickness increases, so will background tissue fluorescence making it important to include “secondary only” or “no antibody” controls to help distinguish signal from noise. New protocols for improved imaging of thick sections are available (Kupferschmidt et al., 2015) and may be useful for certain applications.

Alternate Protocol 1 is a standard protocol for tissue collection and lysate preparation. A common problem encountered is low protein yield from specific brain regions. To improve protein concentration, consider pooling two-hemispheres and/or making sub dissections from multiple 1-mm slices (or generate thicker slices). Alternatives to the fresh sub dissections (e.g., tissue punches from frozen tissue on a cryostat) are discussed minimally here, but exist in other protocols. There are a variety of issues that can arise in the course of western blotting, all of which are well addressed in alternate protocols.

Anticipated Results

These protocols should enable delivery and validation of either AAV or HSV constructs into mouse brain. Depending on considerations addressed previously (serotype/tropism, promoter selection, transgene of interest), stereotaxic infusion of high titer virus should produce robust expression within 2–3 weeks and 3–5 days of delivery for AAV and HSV, respectively. AAV expression is expected to persist for several months *in vivo* (McCown et al., 1996; Kaplitt et al., 1994), whereas HSV expression, with the exception of newly developed retrograde vectors (which have modified promoters that extend the expression to beyond 3 weeks), is generally undetectable within ~10 d of transduction (Neve et al., 2005). Representative images of GFP expression using an AAV and retrograde HSV vector are provided in Figure 3 (A,B and C,D, respectively). With proper construct design, both IHC and western blotting techniques can be used to verify expression and in some circumstances, quantify changes induced by transgene expression. Depending on the experimental purpose (e.g., Cre-mediated recombination, gene over-expression, RNAi, optogenetic molecule delivery, etc.) different control and validation strategies will be required. The described Basic Protocol 3 (validation with IHC) is appropriate for applications where viral infection produces (A) a fluorescent protein or tag suitable for antibody detection, (B) knockdown or knockout of abundant protein with an available specific antibody, or (C) over-expression of a gene of interest detectable by an available specific antibody. Although validation of extent of expression is best achieved using an un-fused fluorescent protein or other marker, it is possible to use the other described situations to confirm extent of expression. While not included here, in some cases procedures for IHC, partnered with appropriate imaging and analysis protocols, can be used to quantify the extent and/or localization of transgene expression. Alternative Protocol 1 (western blotting quantification) is appropriate for applications where the targeted brain region can be precisely obtained by dissection and the

transgene (or fused tag) expressed or knocked down is detectable with an available specific antibody. The tissue collection section of Alternative Protocol 1 produces samples that could be processed for mRNA collection and qPCR quantification, another technique for quantifying the level of transgene expression or mRNA knockdown/knockout.

Time Considerations

Virus aliquoting as described in Basic Protocol 1 requires approximately 30–45 min to complete, including material gathering. Once aliquoted, viruses can be stored long-term at -80°C and stored at -20°C or on wet ice during active use.

Setting up the surgery area described in Basic Protocol 2 takes approximately 30 min. The stereotaxic surgery itself can take between 45–90 min to complete from induction of anesthesia to removal of the mouse from the stereotaxic frame. Teardown and cleanup of the surgery area requires an additional 30 min. Recovery from ketamine/xylazine anesthesia is variable, but most mice will be fully alert within 2 hr of anesthesia induction. Mice should be allowed to recover, with daily monitoring for health, for 7–10 days prior to clip removal. Maximal expression of AAVs tends to take 2–3 weeks *in vivo* (see Figure 3A) and is then maintained for long durations (we routinely run experiments for up to 4 months after initial infection, see Figure 3B). Peak HSV expression occurs ~3–5 days post injections and fully dissipates by ~10 days, with the exception of long-term HSV vectors, which are retrogradely transported and stably expressed for prolonged periods (see Figure 3C, D). Expression time will be dependent on virus and construct design and should be empirically determined (see Support Protocol 1).

The IHC verification described in Basic Protocol 3 takes approximately 2–3 days, depending on the duration and number of antibody exposures. On the first day, sections should be blocked and placed into primary antibody solution overnight. On the second day, sections should be exposed to secondary solutions unless additional overnight primary exposures are required. Also on the second day (if feasible), sections should be mounted, dried, and cover slipped. Mounting and cover slipping takes 30–60 min depending on the number of slices to be mounted. Once cover slipped, slides can be stored away from light but imaged within a week to ensure integrity of the fluorescent signal. Imaging to verify placement takes approximately 10 min per slide, depending on the number of brain regions and the extent of documentation.

The western blotting verification described in Alternate Protocol 1 takes approximately 2–3 days. Tissue is collected on the first day, after which it can be stored at -80°C or processed immediately into lysate. Lysate preparation and quantification takes approximately 2–3 hr, depending on the number of samples and the protein quantification assay. Electrophoresis takes approximately 90 min, and protein transfer can be completed within 1–2 hrs or overnight at a lower voltage. Antibody exposure after transfer can take 1–2 days (or more) depending on the optimized exposure times for each primary antibody. Quantification and analysis using the LiCor two-color detection system takes 30 mins–1 hr.

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Figure 1. Materials for Basic Protocol 2: Stereotaxic Surgery

A. Work-space for stereotaxic surgery. B. Mounted Syringes. C. Close-up of mounted syringes to highlight placement of mounting. D. Close-up of syringe body, note plunger bottom and liquid line.

Figure 2. Materials for Basic Protocol 3: IHC Verification

A. Work-space for IHC protocol. Clockwise L to R: 24-well plate, 1X PBS, Slide Container, Slide Rack, Pencil, Coverslips and Slides, Fine Brush and Glass Hook, Petri-dish, Ethanol-resistant Marker. Not Shown: Disposable Transfer Pipette, DPX Mountant, and Ethanol Solutions B. Close-up of Glass Pipette with Hook and thin trimmed paintbrushes.

Figure 3. Representative Images of AAV- and HSV-mediated Gene Expression In Vivo

A. Antibody stained AAV2-EGFP signal 4 months after intra-NAC infusions of 0.6 μ l of high titer virus B. Antibody stained AAV2-EGFP signal 4 weeks after intra-NAC infusions of 0.6 μ l of high titer virus. C. Minimal expression of GFP at injection site 19 d following intra-NAC shell infusions of a long-term, retrogradely transported HSV-GFP vector using the TH promoter. D. Robust expression of GFP in the VTA 19 d following infusions of TH-HSV-GFP into intra-Nac shell.

Commonly Used Stereotaxic Coordinates for Transgene Delivery into the Adult Mouse Brain

Table 1

Table 4.37.1 Commonly Used Stereotaxic Coordinates

Brain region	A/P	M/L	D/V	Angle	Notes	Source
Bed nucleus of the stria terminalis	+0.14	±0.9	-4.8 ¹	0	AAV5	Jennings et al. (2013)
Basolateral amygdala	-1.4	±3.2	-3.6 ²	0	AAV5	Yan Li
Central amygdala	-1.25	±2.7	-4.25 ¹	0	HSV D/V coordinate refers to depth of injector needle following cannulation	Crock et al. (2012)
Dentate gyrus	-2.0	±1.2	-2.4 ¹	0		Derrick Balu
Dorsal hippocampus	-1.9	±1.6	-1.6 ¹	0	AAVs 1, 2, 5, 6, 8, 9 with differential spread	Aschaeur et al. (2013)
Habenula, lateral	-1.7	±0.48	-3.34 ¹	0	AAV	Stamatakis and Stuber (2012)
Infralimbic cortex	+1.7	±0.4	-2.6 ³	0	AAV5, glass capillary	Cho et al. (2013)
Medial prefrontal cortex	+1.9	±0.3	-2.5 ²	0		Pascoli et al. (2012)
Nucleus accumbens	+1.6	±1.5	-4.4 ¹	10	AAV2/2	Smith et al. (2014)
Nucleus accumbens shell	+1.2	±0.5	-4.9 ¹	0	Long-term HSV, retrograde	Audrey Wells
Orbital cortex	+2.6	±1.0	-1.7/2 ³	0	AAV2/1, injections made at both depths	Hooks et al. (2013)
Prefrontal cortex—dorsomedial	+0.8	±0.3	-1.75 ¹	0	HSV	Vetere et al. (2011)
Prefrontal cortex—ventromedial	+1.75	±0.75	-4.75 ¹	15	Glass pipette recommended	Makoto Taniguchi
Prelimbic prefrontal cortex	+1.8	±0.4	-1.5 ²	0	AAV5	Yan Li
Striatum	+0.9	±1.5	-3.2 ¹	0	AAVs 1, 2, 5, 6, 8, 9 with differential spread	Aschaeur et al. (2013)
Thalamus—motor	-0.45	±0.85	-3.25 ³	0	AAV2/1	Hooks et al. (2013)
Thalamus—sensory	-1.2	±1.3	-2.75 ³	0	AAV2/1	Hooks et al. (2013)
Ventral tegmental area	-3.2	±0.4	-4.48 ²	0	AAV5	Kupferschmidt et al. (2015)

Coordinates are provided for the A/P (anterior/posterior), M/L (medial/lateral), and D/V (dorsal/ventral) axes and are relative to bregma.

¹Indicates D/V measurement taken from skull.

²Indicates D/V measurement taken from dura matter.

Indicates D/V measurement taken from pia matter. In some cases, this information is not provided in the literature; thus, it is critical to verify the accuracy of new coordinates with dye experiments before injecting virus.³