Recombinant Adenovirus in Neurobiology

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Abstract

Recombinant adenoviruses have been extensively applied in basic research and gene therapy. Recently, there is an increase in neurobiological studies using recombinant adenovirus technology to manipulate gene expression in neurological systems. Recombinant adenovirus technology provides a useful tool for investigating the function of a gene of interest, neurocircuitries and the delivery of genes for therapy. In order to take full advantage of this technique, it is important to understand the strategies for the design and application of the virus. The goal of this chapter is to provide a practical protocol on the application of recombinant adenovirus for neurobiological studies. A simplified recombinant adenoviral system, AdEasy, will be applied to generate recombinant adenoviruses for the expression and knockdown of the gene of interest. We discuss the construction of vectors for gene expression and siRNAs, the generation of adenoviral recombinants, and the production of high-titer adenovirus. Furthermore, a procedure for injection of the adenovirus using a stereotaxic apparatus is discussed.

Key words Adenovirus, siRNA, AdEasy, Brain, Stereotaxic injection

1 Introduction

Recombinant adenovirus has been used for gene therapy for cancer and other diseases (see reviews [1–4]). On the other hand, the recombinant adenoviral systems provide useful tools in biomedical research to manipulate expression of genes of interest both in vivo and ex vivo. Due to the ease of production and efficient infection rate, recombinant adenovirus systems have been widely applied in the neurobiological studies for determining the function of genes of interest. For example, we studied the effects of serotonin 1A (5-HT1A) and serotonin 2C (5-HT2C) receptors in the amygdala on anxiety-like behaviors in mice using recombinant adenoviral systems [5]. Applications of recombinant adenovirus include over-expression, knockdown, or inducible knock-in or knockout of target genes. Injection of recombinant adenovirus containing a gene of interest into specific brain region provides a useful tool to study the function of the gene of interest in the brain regions. By including a cell-specific promoter in the recombinant adenoviral
construct, one can control the expression of the target gene to a specific cell type [5, 6]. On the other hand, administration of recombinant adenovirus containing antisense sequences or siRNAs of a target gene can reduce the expression of the gene in the specific brain region and may be cell type specific [7, 8]. Administration of recombinant adenovirus containing sequence for inducing protein, such as Cre DNA recombinase, into inducible knockout or knock-in mice with same inducible system, such as a Cre–loxP, one can activate the inducible systems in specific brain regions and at specific developmental periods [9, 10]. This approach can be used to not only conduct inducible knockout or knock-in in selected brain regions but also can avoid the need to cross-breed the inducible knockout or knock-in mice with CRE transgenic mice that could be lethal. Furthermore, delivery of a gene, which is a neuronal tracer, such as barley lectin or tetanus toxin fragment C, to specific brain regions using recombinant adenovirus greatly facilitates studies on anatomic neurocircuits because the tracers can be controlled by promoters to selectively express in the neurons of interest [11].

One of the most commonly used recombinant adenoviral systems is the AdEasy system [12–14]. The AdEasy system contains shuttle vectors and an AdEasy-1 viral vector. Compared to other viral systems, the AdEasy is a simple and highly efficient system. It contains two vectors, a shuttle vector and a viral vector. Both vectors can be amplified in Escherichia coli, which makes production of the construct and recombinant the virus easier. In the AdEasy-1 vector, E1 and E3 domains are deleted. Therefore, the toxicity of the AdEasy-1-derived recombinant adenovirus is reduced. However, the toxicity of recombinant adenovirus is higher than other viral systems such as adeno-associate virus. Another feature of the adenovirus is limited spread of the virus, which is particularly useful for manipulating gene expression in selective brain regions [6]. In contrast, the recombinant adenovirus is not suitable for alterations of gene expression in the whole brain. Since recombinant adenovirus is not able to proliferate, it dies when the cells propagate. Thus, the infection of proliferative cells, such as astrocytes, usually last for 2–3 weeks. However, because neurons do not proliferation, the viral infection lasts much longer in neurons [6]. Therefore, although recombinant adenovirus has similar infection efficiency for astrocytes and neurons, most infected cells are neurons when a long-term treatment approach is used. Furthermore, recombinant adenoviruses induce only transient expression. It should be noted with caution that the protein expressed by the virus may not undergo physiological and pathological processes similar to endogenous proteins. This is especially important for posttranslational modifications, which may not occur in virally expressed proteins in cell lines. All together, recombinant adenovirus is a useful tool for neurobiological research once we understand the features of the AdEasy systems.
Recently, a modified AdEasy system has been used to express small interfering RNAs (siRNAs) for silencing of a gene of interest. Luo et al. developed an AdEasy-based siRNA expression system \[15\]. Using a screening vector, one can first identify siRNAs that are able to inhibit the expression of the target gene. Once siRNAs are identified, they can be inserted in a shuttle vector as a DNA oligo-nucleotide cassette that can be recombined into AdEasy viral vector. This AdEasy-based siRNA system provides a high-efficiency siRNA deliver system, especially, for in vivo studies. Furthermore, using DNA oligonucleotides as siRNAs is much cheaper than the cost for synthesis of siRNAs.

Since the protocol for generation of recombinant adenovirus has been discussed extensively \[12–14\], in this chapter, we focus on the applications with AdEasy-1-based systems in neurobiological research.

### 2 Materials

#### 2.1 Cloning System

RT-PCR can be used to amplify the sequence of the gene of interest. Common cloning techniques can be used to insert the sequence into the shuttle vector.

#### 2.2 AdEasy-1 System

**Shuttle Vectors**

Shuttle vectors are used for constructing the target sequences so that it can be recombined into adenoviral vectors. The shuttle vectors contain two fragments of the viral backbone that are located in the both sides of the multiple cloning site (MCS) (right and left arms). These right and left arms facilitate the recombination of the shuttle vector into the viral vector. There are four shuttle vectors available in AdEasy-1 system: pShuttle, pShuttle-CMV, pAdTrack, and pAdTrack-CMV. The pAdTrack and pAdTrack-CMV contain a GFP gene that is controlled by a CMV promoter (cytomegalovirus promoter). Thus, the recombinant adenovirus prepared with these shuttle vectors expresses GFP, which assists in visualization of viral expression. The pShuttle-CMV and pAdTrack-CMV contain a CMV promoter at 5′ of the MCS and a SV40 poly A tail at 3′ of the MCS, resulting in CMV promoter-controlled expression of the gene of interest.

**AdEasy-1 Vector**

The adenoviral vector is used to generate adenovirus after recombination with shuttle vector. The E1 and E3 domains of the adenovirus are deleted in AdEasy-1. Thus, reproduction of the recombinant adenovirus requires a cell line containing an E1 domain such as HEK 293 and 291 cells.
To generate recombinant adenovirus containing siRNA, two vectors were developed, one for identification of siRNA (pSOS-HUS) and one, a shuttle vector, for recombination of siRNA into adenovirus (pSES-HUS) [15].

**pSOS-HUS vector** is designed for identifying siRNAs for a gene of interest. The vector contains an siRNA site that is controlled by U6 and H1 promoters, and a target gene site (MCS) that is at 3′ of an eGFP sequence and linked to the eGFP with an IRES promoter, resulting in the expression of eGFP and the gene of interest, whereby they are transcribed together but are translated separately. When an siRNA binds to mRNA of the gene of interest and consequently results in degradation of the mRNA, the eGFP mRNA is also degraded. Therefore, the expression of eGFP is negatively correlated to the effect of the siRNA in the SOS-HUS. The design to include both siRNA and the sequence of the gene of interest in one vector results in transfection of the siRNA and gene of interest simultaneously.

**pSES-HUS** is a shuttle vector for siRNA. The siRNA site is controlled by U6 and H1 promoters. A double-stranded DNA oligo with the siRNA sequence is inserted between Sfi I sites. Using a DNA oligo cassette significantly reduces the cost relative to the cost for siRNA. The pSES-HUS contains an RFP sequence that assists in visualization of the expression of the recombinant adenovirus.

**BJ5183 Cells Containing AdEasy-1 Vector (AdEasier or BJ5183-Ad-1 Cells)**

BJ5183 cells have high capability for homologous recombination and thus are used for recombination of the shuttle vector and AdEasy-1 vector. However, these cells have a relatively low transformation efficiency. The success rate is low when both shuttle vector and AdEasy-1 vector have to be transfected together and then are recombined. A more recent approach is to first transf ect AdEasy-1 into BJ5183 cells and then to transfect the shuttle vector into BJ5183 containing AdEasy-1. This approach for obtaining recombinant adenovirus is much more efficient. BJ5183 cells containing the AdEasy-1 vector are called AdEasier or BJ5183-Ad-1 cells (Stratagene).

**HEK 293 or 291 Cells**

HEK 293 and 291 cells contain an E1 domain of adenovirus that is required for viral propagation. Thus, HEK 293 or 291 cells are used to generate recombinant adenovirus.

### 2.3 siRNA Recombinant Adenovirus System

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Because of the low transformation efficiency of BJ 5183 cells and large size of AdEasy1 vectors, electroporation is highly recommended for generating adenovirus recombinants. In general, electroporation is carried out under the condition of 2.5 kV, 200 Ω, and 25 μFD in an ice-cold 2 mm cuvette.

*Electroporator:* Bio-Rad Micropulser or other electroporators.

A *stereotaxic apparatus* for animals, such as rats and mice, is required for injection of high-titer recombinant adenovirus into selected brain regions.

*Syringe pump* for control the injection rate and volume. Syringe pumps, such as a two-syringe push–pull pump or microsyringe pump with four-channel microcontroller from Would Precision Instruments (Sarasota, FL).

10 μl or 25 μl *Hamilton syringes*.

*Injector* (33 gage, C315I, Plastics One Inc., Roanoke, VA), Guide cannulas and PE50 tubing.

*Slide-A-Lyzer mini dialysis unit* (10,000 MWCO, No. 69570, PIERCE).

### 3 Methods

#### 3.1 Strategies for the Design of Recombinant Adenovirus

In order to design a recombinant adenovirus suitable for your research purposes, it is critical to choose the most appropriate shuttle vector.

As listed in the material section, there are four shuttle vectors in the AdEasy system. pAd-track vectors (with and without CMV promoter) contain a GFP sequence that facilities visualizing viral expression during generation of the virus and for various applications. Furthermore, GFP is a neuronal tracer that can move along axons of infected neurons, which may be beneficial for studies on neurocircuitries. However, the inclusion of GFP sequence reduces the capacity for insertion of a gene of interest. Thus, If an insert is larger than 5.9 kb, pShuttle vectors (maximal insert size = 6.6 kb) should be used. Additionally, the fluorescence of GFP may interrupt endpoint measurements such as immunohistochemistry and Ca²⁺ release assays (*see Note 1*).

Another consideration is whether to use vectors with CMV promoter or not. pShuttle-CMV and pAd-track-CMV vectors contain a CMV promoter that controls the expression of the gene of interest. CMV is a human viral promoter with a high efficiency for viral expression. The CMV shuttle victors also contain SV40 poly A tail. These structures make construction of the adenovirus much easier. However, the CMV promoter drives nonselective expression of the gene of interest. The gene expresses in all of the infected cells regardless whether it is endogenous expressed in
that cell type. Alternatively, the shuttle vectors without the CMV promoter allow for the addition of a cell-type-selective promoter and, thus, express the gene of interest in specific type of cells. We have successfully used promoters for 5-HT$_{1A}$ receptors, 5-HT$_{2C}$ receptors, and GDA67 to control the expression of the gene of interest in select neurons [5, 6]. The drawback to the approach is that the cell-type-selective promoters are usually less efficient resulting in low expression levels of the gene of interest.

Two approaches can be used to reduce gene expression, antisense sequences and siRNA. Luo et al. [15] developed vectors for identification and insertion of siRNA for a gene of interest, which can be then be easily used for recombination with the AdEasy1 system. Since siRNA only binds to its target mRNA, it selectively knocks down the expression of the gene of interest. However, because not all of the siRNA candidates identified by software are able to knockdown gene expression, candidate siRNAs can be tested first using SOS-HUS vectors with ex vivo systems. Moreover, it is necessary to generate a few siRNA viruses [2–4] to ensure knockdown in vivo. Additionally, mismatch control sequences for the siRNA need to be prepared, resulting in the need for generation of four to eight recombinant adenoviruses for the knockdown one gene. On the other hand, the antisense approach may be less selective, especially, if the CMV promoter is used. Since, the specific antisense sequence that inhibits the gene expression is usually not available, we used full sequence of coding region controlled by cell-type-selective promoter. In this case, an adenovirus with shuttle vector alone was used as a control. Thus, the antisense approach requires less work for development of the virus. From our experience, the efficiency of knockdown is not significantly different between the antisense and siRNA approaches.

As Fig. 1 shows, the procedure for generation of recombinant adenovirus includes cloning the gene of interest, constructing a shuttle vector and recombining the shuttle vector into an AdEasy1 vector. The detailed protocol for the procedures can be found in several resources [12–14]. In this chapter, we briefly discuss these procedures.

### 3.2 Generation of Recombinant Adenovirus

#### 3.2.1 Constructing Shuttle Vectors

**Constructing pShuttle- and pAdTrack-Based Shuttle Vectors**

Cloning sequences of interest gene: since all of the techniques used in this step are common molecular techniques, multiple approaches can be used to obtain the clones. Here, we describe a common procedure to clone sequences required.

1. Amplification of the sequence using PCR: the coding region of the gene of interest can be amplified from cDNA, whereas the promoter region should be amplified from DNA. Several tips for primer design are listed in **Note 2**.
2. The PCR products can be directly inserted into shuttle vector after digestion with restriction enzymes. An alternative approach is first to insert the products into a PCR vector such as TOPO pCR II. After a miniprep, correct colons can be selected by restriction digestion and sequencing. Although this approach adds a cloning step, it provides plasmid for higher efficient digestion of the insert. Furthermore, most PCR vectors contain common promoters for sequencing, so that the insert can be sequenced at this step.

*Ligation of insert into the shuttle vector:* after digestion of insert and shuttle vector with same restriction enzymes, the insert can be ligated into the shuttle vector. When the pShuttle and pAd-track vectors are used, a promoter and a polyA tail should also be ligated into 5’ and 3’ of the target gene sequence, respectively. Confirming the expression of the gene of interest by transient transfection of shuttle vectors into a cell line before further generation of recombinant adenovirus is recommended. As described in Note 2, the expression of the gene of interest may require specific sequences, such as noncoding regions, not only Kozak consensus sequences.
Another application of recombinant adenovirus is to generate siRNA for the gene of interest so that the expression of the gene can be reduced. The vectors to generate the recombinant adenovirus containing siRNA were developed by Luo et al. [15]. Two vectors are involved in the procedure: pSOS-HUS is used to identify siRNA that reduce gene expression and the pSES-HUS vector which is a shuttle vector for production of the recombinant adenovirus. Thus, two steps are included in the procedure.

**Identification of siRNA for target genes:** Since siRNA is gene specific, it is necessary to identify siRNA before generation of the adenovirus. Candidate siRNA sequences can be found using one of several online programs, such as Dharmaco’s siDESIGN (http://www.dharmacon.com/sidesign/) and Invitrogen’s BLOCK-iT™ RNAi Designer (http://www.rnaidesigner.invitrogen.com/rnairexpress). The test for the siRNA can start with four to five siRNA candidates. As described by Luo et al. [15], DNA oligos with siRNA sequences, plus an extra “A” at 5′ and four extra “Ts” at 3′ of sense and antisense oligos can be synthesized. A sense sequence of target gene is inserted into multiple cloning sites of pSOS-HUS. A DNA oligo cassette with sense and antisense sequences of siRNA is generated and inserted into SfiI sites of pSOS-HUS containing a target gene as described by Luo et al. [15].

After confirming the sequences of the inserts, the pSOS-HUS containing target gene and siRNA sequence (SOS-siGENE) can be transfected in HEK 293 cells in 12-well plate using standard LipofectAMINE approach. A pSOS-HUS containing the target gene alone is used as a control. GFP expression is observed under a fluorescent microscope on constitutive days after the transfection. The GFP-positive cells are usually not significantly different between the siRNA and control constructs 1–2 days after transfection. However, an siRNA-induced reduction in the number and brightness of GFP-positive cells is usually observed on the 3rd day. The reduction is more extensive 4–5 days after transfection. Thus, these sequences can be selected as siRNA to generate recombinant adenovirus.

Once the siRNA sequences are identified, the oligo cassettes of these sequences can be inserted into pSES-HUS, an siRNA shuttle vector for adenovirus production. The oligo cassettes of siRNA are ligated into SfiI digested sites. The ligated constructs are selected by digestion with NotI or SfiI after the ligation that can cut self-ligated pSES-HUS. After plating the ligation solution in agar plates, the positive colonies are selected by colony PCR using a U6 forward primer and antisense oligo of the cassette. The plasmids of the positive colonies are further sequenced with a U6 forward primer to confirm the sequence of the siRNA. The correct pSES-HUS–siRNA shuttle vectors can be used to generate recombinant adenovirus as described below.
Recombination of shuttle vector into AdEasy-1 vector. The shuttle vector containing the sequence of the gene of interest is linearized by digestion with Pme I (or Eco RI or Bst1107 I, if the insert contains Pme I). The linearized shuttle vector is then transformed into AdEasier cells, whereby the shuttle vector recombines into the AdEasy1 vector. The recombinant adenoviral colonies are then selected by kanamycin, since shuttle vectors are kanamycin resistant, while AdEasy vectors are ampicillin resistant. The smallest colonies should then be picked and minipreps is prepared. The size of supercoiled plasmids is checked using a 0.7 % agarose gel. The recombinant adenoviral plasmids run slower than 12 kb, whereas shuttle vectors alone run about 5 kb [14]. The candidate plasmids can be further digested by Pac I, which should yield a large fragment (>33 kb) and a smaller fragment (3 or 4.5 kb) [14]. The correct colonies are transformed into a strain of E. coli cell that has high plasmid propagation but not recombination features, such as DH10B, to generate more plasmids (see Note 3). The plasmids are further confirmed using restriction enzyme digestion, such as with Hind III, and PCR. The confirmed plasmids are further used for generation of high-titer recombinant adenovirus.

Generation of high-titer recombinant adenovirus: after linearizing with Pac I, the recombinant adenoviral plasmids are transfected into HEK 293 cells as described by He et al. [12–14]. Briefly, the linearized plasmids are transfected into HEK 293 cells using a standard transfection approach such as LipofactAMINE. Two to three weeks after the transfection, when all of cells have become round and 50 % of the cells are detached (cytopathic effect, CPE), the cells can be collected by scraping them with a cell scraper (do not use trypsin). The cell lysate is then used to infection more cells to generate high-titer recombinant adenovirus. If it is desired, the supernatant (media) can also be used to further infect cells, which can accelerate the production of high-titer adenovirus.

To obtain high-titer adenovirus, 15–20 75 cm flasks of viral-infected HEK 293 cells are needed, which may require two to four rounds of infections starting from the viral lysates (may be media) from transfected cells above (see Note 4). The viral lysates are further used to infect HEK 293 cells until infected HEK 293 cells are sufficient for preparation of high-titer adenovirus (15–20 flasks). High-titer adenovirus is prepared as described in previous protocols [13, 14] (see Note 5).

Check titer of recombinant adenovirus: several methods can be used to check the titer of recombinant adenovirus as described by Luo et al. [14]. We have used GFP (or RFP) expression to check the titer of adenovirus. HEK 293 cells are plated in 12-well plates with 1 ml medium per well. Next day, the high-titer recombinant adenovirus is serially diluted using tenfold dilution intervals. Ten microliters of diluted viral solution (usually 10^3 and 10^8 dilutions) are added into each well, respectively. The infected cells (GFP or
RFP positive) are observed 24 h later. The number of GFP-positive cells is counted in the well with highest viral dilution. The titer of the virus can be calculated as:

\[
\text{Infective viral particles} / \text{ml} = \frac{\# \text{ of infected cells} \times \text{viral dilution in the well} \times 1,000}{10}
\]

### 3.3 Manipulation of Gene Expression in the Brain Using Recombinant Adenovirus

Once generated, the high-titer recombinant adenovirus can be used to manipulate gene expression in vivo and ex vivo. In this chapter, we focus on the approach to alter the expression of a target gene in specific brain regions using recombinant adenovirus.

#### Preparation for Adenovirus Injection

High-titer adenovirus is collected by CsCl gradient centrifugation and stored in a high-salt glycerol solution that can cause tissue damage. Therefore, it is necessary to remove the remaining CsCl, high salt and glycerol before injection into the brain. On the day of injection, the viral stock solution is dialyzed by transferring the viral stock into the bottom of a slide-A-Lyzer mini dialysis unit (10,000 MWCO, PIERCE) that is then placed in a flotation device (regular vial floating plates can also be used). The dialysis units are floated in saline (500–1,000 ml) with slow speed stirring at 4 °C for >30 min. Collect the viral solution (the volume may be slightly increased) into a clean vial and further dilute 1:1 with saline. The viral solution is ready to use and is placed in ice (see Note 6).

#### Assembling the Stereotaxic Injector

To inject recombinant adenovirus, it is essential to control the speed of injection so that the viral solution can diffuse into tissue and reduce the amount that backs up into the needle track. Thus, we use a syringe pump to control the rate of injection. An internal injector (33 gage, C315I, Plastics One Inc., Roanoke, VA) is connected to a 25-μl Hamilton syringe with PE50 tubing. The syringe is placed on an injection pump to control the injection rate. The injector is held by a 26-gage guide cannula with a tubing length of 2 mm below the pedestal, which is then mounted on the stereotaxic device (Fig. 2). Before injection, the PE50 tubing is filled with ddH₂O to reduce resistant. An air bubble is left at the injector end to separate recombinant adenovirus from ddH₂O. For bilateral injection, two internal injectors are held by a double guide cannula (Fig. 2a). An alternative is to directly inject through a 10-μl Hamilton syringe that is held by a microsyringe pump with four channel microcontroller (Fig. 2b).
Fig. 2 Assembling stereotaxic injector: (A) stereotaxic injector for bilateral injection. A double internal injector (1) is held by a double guide cannula (2) that is mounted on a stereotaxic apparatus via a cannula holder (3). The internal injector is connected to Hamilton syringe (5) with PE50 tubing (4). The Hamilton syringes are controlled by a syringe injection pump (6). (a) Enlarged picture for the injector assembling [white box in picture (A)]. 2: Single and double guide cannulas used for hold internal injector. (B) Alternative assembling stereotaxic injector for unilateral injection. A microsyringe pump (7) with a Hamilton syringe (5) is mounted on stereotaxic apparatus. The microsyringe pump is controlled by a four-channel microcontroller (8) (World Precision Instruments, Sarasota, FL)

Injection of Recombinant Adenovirus into Specific Brain Regions

An animal is anesthetized and placed on a stereotaxic apparatus. After an incision, the tip of injector (one of the injectors for bilateral injection) is aligned with bregma and all of the coordinates are rezeroed.

(continued)
From our experience, the viral-induced gene expression can be observed 3 days after injection and reached a maximum 5–7 days after the injection. Although higher injection volumes of recombinant adenovirus produce higher effects of gene manipulation, it also causes higher toxic effects. We usually use 1–2 μl of high-titer virus, depending on the size of the region of interest and the titer of the virus.

Using recombinant adenovirus with a sense gene sequence, the expression of the gene of interest can be increased up to three-to fivefolds over endogenous protein levels (control). On the other hand, recombinant adenovirus with antisense or siRNAs can reduce the gene expression to more than 50 % when the tissue punched from viral-infected area is measured relative to contralateral control. However, in experiments in which recombinant adenoviruses are bilaterally injected and tissue in the target region is collected, knockdown rate for a group of animal is usually about 30–40 % relative to a control viral group. This could be due to the variation of injection between the animals and even between both sites of the brain regions, which masks the reduction of gene expression induced by adenovirus (see Note 9).
4 Notes

1. It should be noted that the GFP (or RFP) is expressed independently to the sequence of interest. Since it is controlled by a CMV promoter, the GFP is nonspecifically expressed in virally infected cells. Thus, GFP can be used as index for viral infection. On the other hand, the expression of interest genes, especially those controlled by specific promoter, may be expressed in selected cell types but not all of virally infected cells.

2. When designing primers for amplification of the gene of interest, it is suggested that (1) use cDNA and DNA from same species of animal to be used in the study; (2) it is important to include an RNA polymerase binding site, such as a Kozak consensus sequence (ACCAUGG or extended form: GCCGCCACCAUGG) at 5′ of coding region. Here, AUG is starting codon (ATG for DNA). Without an RNA polymerase binding site, the translation may not be processed. In some cases, even with Kozak sequence, the gene may still not be expressed. We found that including 5′ noncodon region may facilitate gene expression. (3) It is recommended to include restriction enzyme sites in primers, so that it can be digested by restriction enzymes for insertion into shuttle vectors.

3. Do not regrow BJ5183 cells containing recombinant adenovirus plasmid because of the recombination and rearrangement features of BJ 5183 cells.

4. In order to obtain high-titer virus, it is critical that infected cells reach the desired CPE (i.e., all of cells become round and 50 % of the cells are detached) in 3–5 days. Shorter or longer times required to reach the desired CPE reduce the yield of recombinant adenovirus, resulting in a faint band or no detected band is after CsCl gradient centrifugation.

5. In contrast with pAdTrack (SES-HUS) vectors in which GFP (or RFP) can be used to monitor the transfection and infection, pShuttle vectors cannot be monitored except with the CPE. Therefore, it is critical to verify the insert in the viral lysate after transfection and infection.

6. Dialyzed recombinant adenovirus is not stable at room temperature. It can be inactivated in 30 min at room temperature, whereas it can stay stable in ice for 3–5 h. Thus, it is essential to keep the virus in ice until injection. Also, if the injection lasts for more than 10 min, it is recommended to take only 5–10 μl of the viral solution at a time and refill the solution for each injection.

7. The coordinates of stereotaxic injection are based on brain atlases such as for mice or rats [16, 17]. However, the effects
of the pressure from injector on the brain should be considered. During injection, the pressure of the needle can push the brain moving to a more ventral and lateral location, resulting in a change of the coordinates. This particularly occurs when injecting regions are in the ventral region of the brain such as the hypothalamus and amygdala. Thus, the actual dorsal/ventral coordinates are usually greater than those in the atlas (more ventral). Similarly, the injector may enlarge the ventricle when passing through it. Thus, the dorsal–ventral coordinate becomes larger than that in the atlas. Therefore, it is necessary to test the coordinates prior to experiments.

8. To evaluate the effects of recombinant adenovirus, it is essential to collect the tissue with viral infection, not the brain region intended for injection, because it is possible that the actual injection site is different from the desired injection site. We punch the infected tissues out of 300-µm coronal brain sections. It is difficult to observe the fluorescent areas in such thick sections even with dissection of the tissue under a microscope (unless dissecting microscope has a UV light source available). We have used thin sections (20–30 µm) to estimate the position of GFP (RFP) in the brain and dissected the correlated regions from thick sections. The fluorescence on the thick sections is then checked under fluorescent microscope to ensure the GFP (RFP)-positive area is dissected. On the other hand, after the effects of recombinant adenovirus is confirmed, the protein levels in the specific brain regions should be measured to determine the manipulation of target protein in the specific brain regions. In this case, the location of GFP expression should be observed and recorded in brain sections (20–30 µm) before, in the middle of, and after the sections used for collecting the tissues. The animals should be excluded from the study if the GFP expression is not located the desired brain region in sites of the injection.

9. The alterations in protein levels after injection of recombinant adenovirus not only depends on the efficiency of the virus but also depends on the protein turnover rate and feedback regulation. The effect of knockdown of a recombinant adenovirus may not be observed until existing proteins are degraded. On the other hand, the feedback regulation may increase the synthesis of the protein, once the protein level is reduced by the viral treatment. Thus, a time-course study is necessary to determine the optimal period for knockdown. From our experience, most of antisense and siRNAs produce maximal knockdown 5–7 days after the viral treatment.
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