

Sindbis Virus-Mediated In Vivo Expression of Recombinant CREB to Study Gene Function in Neuronal Plasticity and Behavior

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Abstract

Understanding the role of specific genes in diverse brain functions is a major challenge in neuroscience. To address this issue, we have optimized an in vivo transgene expression system based on the sindbis virus. In this chapter, we provide a detailed description of the cloning, production, and in vivo stereotaxic injection of sindbis viral particles expressing proteins of interest. As an example, we describe how this technique can be used to identify the roles of the transcription factor, cAMP-responsive-element binding protein, in the regulation of neuronal plasticity and memory formation.

Key words In vivo gene expression, Sindbis virus, CREB, Neuronal plasticity, Memory, Electrophysiology

1 Introduction

The transcription factor cAMP-responsive-element binding protein (CREB) is involved in the regulation of very diverse cellular functions, including metabolic control, cell proliferation, cell differentiation, and apoptosis. CREB also participates in many processes related to signaling in the nervous system. In fact, the CREB pathway probably represents the best characterized transcriptional cascade in the adult brain.

The convergence of multiple intracellular cascades on CREB positions this transcription factor in an ideal situation to integrate different stimuli and regulate neuronal responses. The activation of CREB by phosphorylation can be triggered in neurons by a wide variety of signaling processes, from an increase of Ca^{2+} through activation of voltage- or ligand-gated channels to an increase in cAMP levels after activation of G-coupled receptors and an activation of receptor tyrosine kinases by growth factors [1, 2]. CREB activation generally results in expression of genes carrying the CRE

sequence in their promoter. The final effect of this activation, i.e., the number and specific identity of CRE-dependent genes that will be turned on, will depend on both the nature of the stimulus and the cellular context [3]. A number of excellent articles have reviewed different aspects of CREB's brain functions [1, 2, 4–6]. In particular, there is strong evidence both in invertebrates and in mammals that CREB is implicated in memory formation. The exact extent of its role, however, is still under intense investigation.

In this chapter, we provide a detailed methodology for how the sindbis virus expression system has been successfully used to express recombinant CREB proteins in the brain *in vivo*. This technique allows for rapid and transient *in vivo* expression of a protein of choice in a temporally and spatially restricted manner. Importantly, the neurons expressing the recombinant proteins are allowed to do so while remaining in their physiological environment in freely behaving animals. We also review how this molecular manipulation can be combined with electrophysiological or behavioral analyses to investigate how CREB regulates neuronal plasticity and memory formation.

2 Materials

In this section, we provide a list of the material necessary for cloning, production, and *in vivo* injection of sindbis viruses. Common reagents and equipment necessary for general molecular biology or cell culture manipulations are not listed.

2.1 Cloning into Sindbis Virus-Based Vectors

1. Sindbis viral vector backbone pSINRep(nsP2S⁷²⁶) or pSINRep(nsP2S⁷²⁶)-IRES-GFP (Fig. 1a) [7]
2. cDNA of protein-of-interest
3. Custom made primers for cloning (see Sect. 3.1.1 and Fig. 2b)
4. PfuI polymerase enzyme and reagents
5. Restriction and ligation enzymes and buffers (e.g., XbaI, SphI, T4 ligase)
6. DNA amplification and purification kits (e.g., QIAprep Spin Miniprep kit and QIAquick PCR purification kit or QIAquick Gel Extraction kit, QIAGEN)
7. Electro-competent or chemically competent *E. coli*
8. Sequencing primers (the pSINREP5 primer is provided in Fig. 1b, which permits sequencing downstream of the multiple cloning site (MCS) of the sindbis virus-based vectors)

2.2 Production of Sindbis Viruses

1. Level 2 biosafety tissue culture room including tissue culture hood and incubator
2. Restriction enzyme for linearization (XhoI or PacI or NotI)

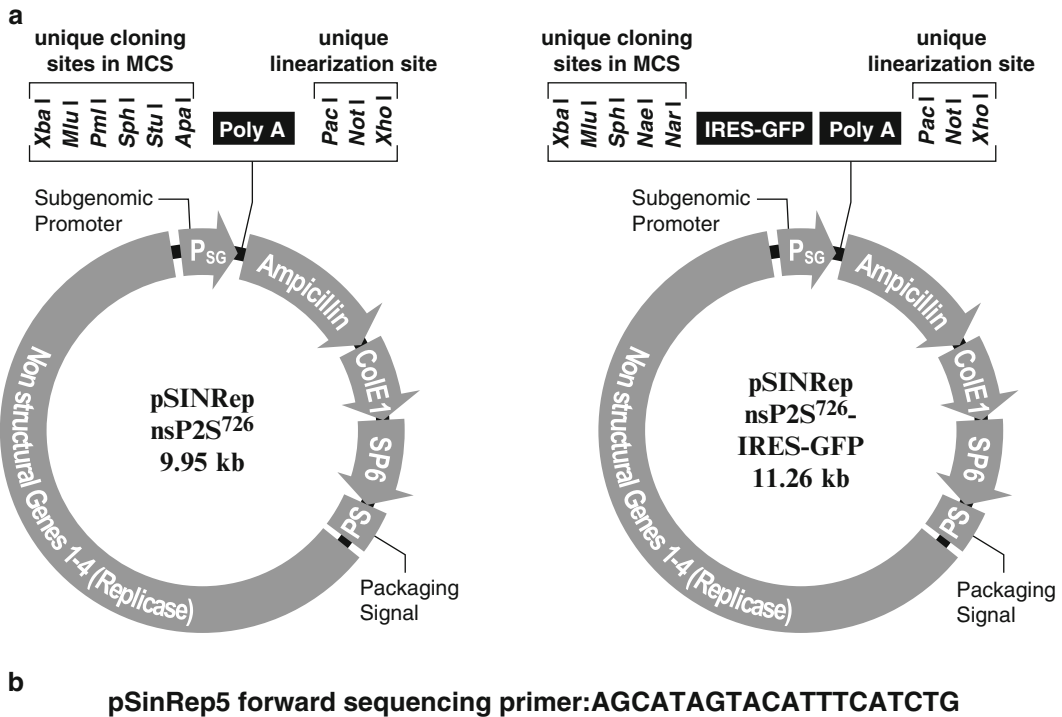
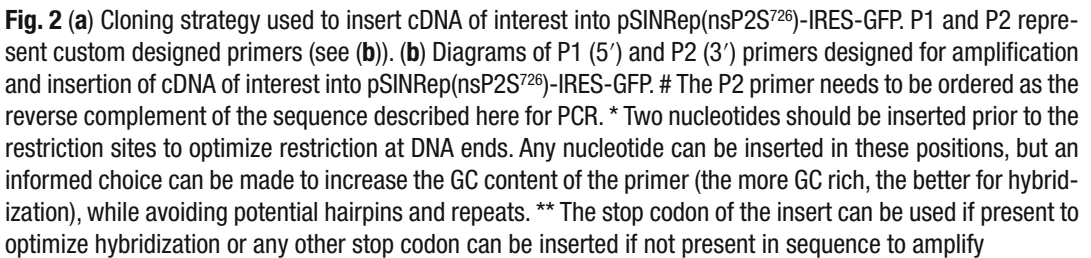


Fig. 1 (a) Vector maps of pSINRep(nsP2S⁷²⁶) and pSINRep(nsP2S⁷²⁶)-IRES-GFP. The unique restriction sites are annotated in the multiple cloning site (MCS) and for linearization of the vector prior to virus production. (b) Sequencing primer pSINREP5 used to sequence insert

3. Helper DH-BB(tRNA/TE12) DNA [7]
4. Cloned recombinant sindbis virus DNA (Sect. 3.1.1)
5. DNA amplification kit (e.g., QIAfilter Plasmid Maxi kit)
6. DNA purification kit (e.g., QIAquick PCR purification kit)
7. In vitro transcription kit (e.g., mMESSAGE mMACHINE® SP6 Kit, cat. No. AM1340, Ambion, Inc, USA)
8. Tissue culture plasticware including 150 mm² plates and T150 mm² flasks with filtered caps
9. Phosphate buffer saline (PBS) with cations, i.e., MgCl₂ and CaCl₂
10. RNase-free PBS without cations
11. Trypsin EDTA
12. αMEM medium with l-glutamine
13. Fetal bovine serum (FBS)
14. Bright field microscope and cell counting instruments
15. Electroporator for mammalian cells (e.g., Biorad Gene Pulser XCell)
16. Biorad electroporation cuvettes (0.4 cm)



17. Beckman ultracentrifuge
18. Beckman rotor SW40Ti (swinging buckets)
19. Beckman ultra-clear centrifuge tubes (cat. no. 344059)

2.3 *In Vivo* Injection of Sindbis Viruses

1. Rat or mouse stereotaxic frame (depending on species to be used for the studies)
2. Microdrill
3. High accuracy pump (e.g., Harvard PHD2000 or KSD legato 110)
4. Five μL injection syringe (e.g., SGE Analytical science cat. no. 5F-HP-0.63/0.47)
5. Injection cannula (e.g., Plastic one cat. no. C315I)
6. Mineral oil
7. PB intramedia polyethylene (PE) tubing (Fisher USA cat. No. 1417011B)
8. Hair clipper
9. One scalpel, two hemostats
10. Surgical suture kit (needle, thread) or Michel suture clips
11. Xylazine hydrochloride, bupivacaine, ketamine hydrochloride and buprenorphine hydrochloride

Notes: The use of a 5 μL SGE injection syringe or similar product is important to optimize the injection system. This syringe has a dual gauge needle, which permits tight fitting of the PE tubing (item 5) to avoid air leaks and loss of pressure. Use of items 4, 5, and 7 together is crucial to obtain reliable pressure for microinjection. Two compounds of item 11 (ketamine and buprenorphine HCl) are classified as potential substances of abuse and their purchase is regulated in several countries. An official authorization might be necessary prior to purchase.

3 Methods

We have chosen the sindbis virus system because it produces the recombinant protein-of-interest rapidly (within 24 h) and is highly neurotropic [8]. The sindbis virus is a member of the alphavirus family, like the Semliki forest virus. These viruses are small-enveloped viruses with single-stranded RNA genomes [9]. The sindbis expression system is a transient expression system in which the sindbis life cycle is exploited to produce recombinant proteins. The first generation of vectors using the sindbis virus backbone (pSINRep-5), in which the insert of interest is cloned, also contained the nonstructural genes, but lacked the structural viral proteins normally necessary to package the RNA into viral particles. These DNA constructs are subsequently used to make genome-length RNA transcripts in vitro (recombinant RNA). Production of replication-deficient infectious viruses is accomplished by transfecting cells with the recombinant capped RNA and a helper RNA that provides the structural proteins in *trans*. Particles released by

the transfected cells contain only the recombinant RNA and are ready to infect new cells for expression studies. These recombinant viruses will undergo only one round of infection as they do not contain the helper RNA which encodes the structural proteins. Expression of the transgene is detected within a day both in vitro and in vivo. More information on the sindbis viral system can be found in [9].

The main advantages of this type of virus in neuroscience are its high neurotropism (preferentially targeting glutamatergic neurons), the strength and speed of transgene expression, and the good diffusion of viral particles in vivo. On the other hand, these viruses lead to cytotoxicity within a few days after infection and are therefore not suitable for long-term expression studies. This is due to the fact that the recombinant RNA, once transfected into cells, promptly recruits the host translational machinery for its own use, resulting in high levels of the desired protein, but at the expense of cell viability (it shuts off gene expression in the host cell). Also, transgene size is limited as packaging becomes problematic if the insert size is more than 4 kb. In vivo investigations using this virus have used a time frame of expression of up to 3 days with success [10].

A new generation of viral backbone vectors was designed to reduce this toxicity [7]. The low-toxicity vector pSINRep(nsP2S⁷²⁶) (Fig. 1a) contains a point mutation in the second nonstructural protein (nsP2), which delays the inhibition of host protein synthesis. Kim et al. [7] also constructed an optimized helper vector (DH-BB(tRNA/TE12)) to use with this new backbone vector for production of particles with low levels of helper RNA packaging and high neuro-specificity of infection.

For identification of infected cells, we have generally used the green fluorescent protein (GFP) and have therefore constructed the pSINRep(nsP2S⁷²⁶)-IRES-GFP vector (Fig. 1a). The internal ribosomal entry site (IRES) allows for co-expression of the protein-of-interest and GFP in the same cells without the need for fusion proteins. This, however, limits the maximum size of the cDNA encoding the protein-of-interest to little more than 2.5 kb.

In this chapter, we provide procedures for cloning, production, and in vivo injection of sindbis viruses in the rat hippocampus. We have, however, not detailed standard molecular and cellular biology techniques (e.g., digest with restriction enzymes, splitting of cells in culture).

3.1 Cloning of Recombinant Sindbis Viruses

In this section, we review the cloning procedure to engineer recombinant sindbis virus vectors. Since each cloning will be unique and designed to fit the compatibility of the insert with the vector, the specific cloning strategy should be adapted in each case. As a guide, we describe the cloning strategy that we generally use to insert the cDNA of proteins of interest into the sindbis virus vector containing IRES-GFP (Fig. 2a). As this method relies on

PCR amplification of the insert with primers that contain suitable restriction sites for cloning into either pSINRep(nsP2S⁷²⁶) or pSINRep(nsP2S⁷²⁶)-IRES-GFP, this procedure should in principle be adaptable for cloning most inserts into these vectors. The main limitation of this cloning is that the sindbis vector cannot host more than a total of about 4 kb of insert (including IRES-GFP); otherwise, its packaging efficiency significantly diminishes. Also, it is necessary that the insert to be cloned does not contain restriction sites used during the cloning or linearization steps. Nevertheless, this cloning strategy can be adapted for other types of cloning using the MCSs available in the sindbis vectors (Fig. 1). We do not detail each step in this cloning (e.g., PCR amplification, restriction digests), but specifics can be found in standard molecular biology manuals and kit manuals.

3.1.1 Cloning of the cDNA of the Protein-of-Interest into pSINRep(nsP2S⁷²⁶)-IRES-GFP

Primers are designed to amplify the 5' and 3' ends of the insert cDNA. A diagram of these primers is depicted in Fig. 2b. The 5' end primer, called here P1, should also contain an XbaI restriction site and a Kozak sequence to optimize initiation of translation of your insert. The 3' end primer, called here P2, should contain a stop and an SphI restriction site. We recommend the use of XbaI and SphI enzymes for cloning in the sindbis vectors as we have proven their efficiency repeatedly during this procedure. If these sites are present in your insert of interest, alternative unique sites in the MCS (Fig. 1) can be selected and introduced using a similar primer design.

- (a) Amplify cDNA of protein-of-interest using the custom-made primers described above. We recommend the use of Pfu polymerase or another high-fidelity DNA polymerase for PCR amplification to minimize mutagenesis.
- (b) Digest both the sindbis virus vector and the PCR product with XbaI and SphI. Optional: the vector can be 5' dephosphorylated using an alkaline phosphatase to prevent self-religation.
- (c) Optional: gel purify or column purify the linearized vector and PCR product to obtain cleaner material for ligation.
- (d) Ligate PCR insert to vector using a T4 ligation kit. It is crucial to respect the ligation procedure by determining the molar ends of both products and calculating the amount of each product to obtain an adequate ratio. Details of this optimization are provided with the ligation kit. A useful control for the cloning procedure is the preparation of a ligation reaction with the vector alone.
- (e) Transform the ligations by electroporation or chemical transformation into *E. coli*. The number of colonies on the vector plus insert plate that should be several fold higher (minimum of ×2) than on the vector alone plate, suggesting successful cloning.

- (f) Check for correct insertion by restriction digest on miniprep DNA (e.g., using an XbaI/SphI restriction digest) or PCR on single colony (e.g., using the custom-designed primers), both of which should display a DNA band of the size of the insert.
- (g) Sequence clone of interest to check for eventual unwanted mutations. The pSINREP5 forward primer is provided in Fig. 1b. Additional primers within the insert may be needed for sequencing the entire insert.

3.2 Production of Sindbis Viruses

During this procedure, we will generate recombinant RNA and helper RNA for transfection in baby hamster kidney (BHK) cells to produce infectious viral particles. We will use purified, linearized pSINRep(nsP2S⁷²⁶) containing our gene of interest (cloned using the procedure in Sect. 3.1) and helper DH-BB(tRNA/TE12) DNA to produce recombinant RNA with an in vitro transcription kit. The recombinant RNA produced must then be capped and have a polyA tail, so it will be treated as a messenger RNA when transfected into the BHK cells. The viral particles produced by the BHK cells will then be harvested and concentrated prior to in vivo injection. The end of this protocol (from Sect. 3.2.2, step k) needs to be performed in a *biosafety level 2* tissue culture room using all the safety procedures designed to work with viruses.

3.2.1 Linearization of DNA and RNA Preparation

- (a) Prepare clean recombinant vector DNA and helper DNA plasmids using a standard DNA purification protocol. An RNase-free purified product is recommended to avoid degradation of your transcripts after in vitro transcription.
- (b) Linearize 5–10 µg of recombinant vector DNA and helper DNA plasmids. Choose an enzyme that has a unique site (XhoI or PacI or NotI) after the PolyA signal and does not cut in the insert (see Fig. 1). The helper DNA should be linearized with XhoI. Each in vitro transcription will require 1 µg of linearized template.
- (c) Purify the linearized plasmids with a DNA purification kit. Resuspend the linearized plasmids in RNase-free TE or water. Optional: run an aliquot of the linearized plasmids on an agarose gel to check its quality and quantity.
- (d) Perform an in vitro transcription reaction for each plasmid using commercially available kits (e.g., mMESSAGE mMACHINE® SP6 Kit).

A typical reaction should yield 10–20 µg of RNA from 1 µg of linearized template. At this point, the RNA can be aliquoted in 10 µL samples and frozen at –80 °C for later use, although use of fresh RNA is best. Optional: check the quality and/or quantity of the RNA by gel electrophoresis and/or by standard quantification measurements.

3.2.2 Transfection of BHK Cells for Virus Production

The transfection procedure is described here for production of one virus. Perform the exact same steps in parallel for each virus to be produced.

- (a) Prepare a 70 % confluent 150 mm² plate of BHK cells per virus. It is best to split cells the day before transfection. BHK cells grow best in α MEM medium supplemented with 5 % FBS and (optional) antibiotics (e.g., streptomycin + penicillin).
- (b) Wash plate with PBS containing cations.
- (c) Collect cells by trypsinization.
- (d) After centrifugation (2 min at 1,000 rpm), discard supernatant and resuspend cells in 10 mL of α MEM without FBS serum.
- (e) Centrifuge again, discard supernatant, and resuspend cells in 10 mL of RNase-free PBS without cations.
- (f) Determine number of cells using a counting chamber.
- (g) Centrifuge cells, discard supernatant, and resuspend cells in RNase-free PBS without cations at a concentration of 10⁷ cells/mL. BHK cells prepared for electroporation must be used immediately and cannot be stored.
- (h) During centrifugation in step g, place a 0.4 cm electroporation cuvette on ice and add 11 mL of α MEM medium + FBS to a sterile filtered top 150 mm² flask.
- (i) Pipette 0.5 mL of the 10⁷ cells/mL suspension in the 0.4 cm cuvette. Then add to the cuvette:
 - 10 μ L of the recombinant sindbis vector RNA (Sect. 3.2.1)
 - 10 μ L helper RNA (Sect. 3.2.1)
 - mix cells and RNAs by tapping the cuvette gently
- (j) Electroporate cuvette: Voltage setting on Biorad Gene Pulser XCell should be
 - Exponential protocol:
 - E: 1,200 V
 - C: 25 microF
 - R: infinite
 - C: 0
 - Pulse once

After each electroporation, the time constant displayed should be ~0.6–1.0 ms. These settings will need to be optimized if using a different size of cuvette or a different electroporator.

- (k) Place cuvette back on ice to minimize damage to electroporated cells. Add 500 μ L α MEM + FBS to resuspend cells in the

cuvette (total volume should be ~1 mL including cell suspension). Transfer this 1 mL suspension to the 150 mm² flask containing 11 mL α MEM + FBS prepared in step h. The total volume of virus suspension should be 12 mL.

- (l) Incubate cells for ~36 h at 37 °C.
- (m) Check the efficiency of transfection after ~18 h by calculating the percentage of GFP expressing cells (if the insert was cloned in the pSINRep(nsP2S⁷²⁶)-IRES-GFP). A minimum of 60 % transfection efficiency is necessary to produce a good titer of virus compatible with in vivo infection experiments. 100 % transfection efficiency can be observed within 36 h if the protocol is optimized. At this point, the cultures produce a very high number of infectious viral particles; therefore, the experimenter must be careful when handling them.

3.2.3 Collecting Virus and Concentration for In Vivo Injection

We are now working with infectious virus particles. It is crucial to wear protective clothing and perform all procedures in a *biosafety level 2* area. All liquid and solid materials that came into contact with the viral solution must be inactivated with hypochlorite before disposal.

- (a) Cool down the Beckman ultracentrifuge and SW40Ti swing-buckets rotor to 4 °C.
- (b) Transfer supernatant from flask (12 mL total) to a sterile 15 mL tube, cap tube and centrifuge at 3,000 rpm for 5 min to remove debris.
- (c) Transfer supernatant in Beckman ultra-clear centrifuge tube and centrifuge at 30,000 rpm for 1 h and 30 min at 4 °C.
- (d) After end of centrifugation, pick up the Beckman tube carefully and remove as much supernatant as possible (try to leave ~200 μ L) without touching the bottom of the tube. The virus particles are concentrated at the bottom of the tube but the pellet is clear and cannot be detected by eye.
- (e) Gently resuspend the invisible virus pellet in the 200 μ L left-over medium with a 1 mL pipette.
- (f) Aliquot 10 μ L of virus into small tubes and store in a -80 °C freezer. We have noticed that freeze-thaw cycles do not significantly affect the virus titer if performed within a few days of each other, but prolonged storage (>1 year) could reduce infection potential.

We have optimized an easy titration procedure to qualitatively evaluate if the viral solution obtained is suitable for in vivo infection. Here is a brief description of this procedure:

1. Plate 100,000 BHK cells/per well in 6 wells of a 24-well plate (in 1 mL of α MEM + FBS) in the morning. A coverslip may be added to each well for more accurate evaluation of infection percentage using a confocal microscope after fixation.
2. After 7 h, infect each well with either 5-, 2.5-, 1-, 0.5-, 0.1-, or 0.01- μ L of the viral solution aliquoted in step f.
3. After 24 h incubation at 37 °C, check for percentage of infection in each well on the live cell using GFP fluorescence (or after fixation and mounting if a coverslip was present in the wells). We have consistently observed that a viral solution that displays strong infection (at least 80 %) even in the 0.1 μ L infected well (sometimes also in the 0.01 μ L well) should provide good in vivo infection.

3.3 In Vivo Injection of Sindbis Viruses in the Rat Hippocampus

We have optimized an in vivo surgery procedure to inject the brain of rats or mice with recombinant sindbis viruses. This procedure is described here for infection of CA1 pyramidal neurons of young adult rats (20–22 postnatal days), but can be adapted to any area of the brain, at any age as well as for infection of mice (see for example [11]).

- (a) Inject rat with an anesthetic by intraperitoneal (IP) injection. We recommend the use of a cocktail of ketamine/xylazine. For juvenile rats, the cocktail consists of 2.4 mL ketamine (100 mg/mL), 1.1 mL xylazine (20 mg/mL), and 6.5 mL sterile saline. We inject it at a dose of 0.1 mL/30 mg of body weight. Anesthesia parameters should be adapted to the age of the animal and the species. For mice, we have also used chloral hydrate as an anesthetic (400 mg/kg) [11].
- (b) While the rat is falling asleep, prepare the pump and tubing that connects the needle placed on the pump to the *Plastic One* cannula. This tubing needs to be filled from beginning to end with mineral oil by capillary action. Do not allow the presence of bubbles within the tube. Make sure that the plunger of the needle is completely inserted into the needle prior to connecting it to the tubing. Connect the tubing to the needle and to the cannula, avoiding the spread of oil within the cannula or the needle.
- (c) Once the animal is asleep, shave the head of the rat with hair clippers and increase local analgesia by injecting bupivacaine (2 mg/kg) at site of incision by local subcutaneous injection.
- (d) Place rat into stereotaxic frame and insert the ear bars. Make sure that the bars are well placed on head. Proper placement can be confirmed if the rat's head can only move in the

forward-backward axis and not sideways. This step is crucial for the procedure to succeed and is difficult to master for beginners. If the head is not correctly positioned, the injection coordinates will be skewed and the intended infection site will most likely be missed.

- (e) Insert mouth holder carefully making sure that it is placed between the teeth. Press the mouth bar onto the head (should be at a level just above the eyes) until the skin on the skull is not loose anymore. Do not press too hard as it could injure the animal.
- (f) Cut an incision on top of the head so as to expose the skull from the top of the mouth bar to the end of the skull. Make sure enough skin is cut to be able to see the Bregma. Place the hemostats to keep the skin well away exposing the skull. The rat is now ready for injection.
- (g) Fill the cannula and tubing with virus solution using the refill (withdraw) function of the pump. Do not allow for bubbles to form during that process (except for the air gap between the mineral oil and the virus solution). For a dual injection (left and right hemispheres), add about 2.5 μL of viral solution. 0.8 μL of PBS may also be added after the viral solution, avoiding air between the two solutions to help with flow (see point k below).
- (h) Place the cannula above the Bregma. Then, elevate the cannula again to avoid contact of cannula with skull that would bend it while traveling to final destination.
- (i) Using Y (vertical/posterior) and X (horizontal/lateral) axes, travel to final destination: The coordinates for injection in a 20–22-day-old rat hippocampus CA1 are:
 X: ± 2.5 mm lateral from Bregma; Y: -4 mm posterior from Bregma
 We have found that keeping the weight of the animal constant yields more accurate injections than solely choosing the animal by age. Also, systematically using the coordinates by carefully locating bregma for each injection is crucial to obtain reliable injections at the desired location. Judging the best place of injection by eye never works reliably.
- (j) Drill hole through the skull superficially without touching the meninges at the site where the cannula will be inserted. Open the dura mater, the outermost of the three layers of the meninges surrounding the brain, locally using the bent end of a needle. If the dura is not pierced, it will prevent smooth insertion of the cannula in the brain and skew the injection.
- (k) Lower the cannula to touch the brain, and start injection using the infusion mode on the pump (0.5 μL per minute)

while lowering the cannula slowly (1 mm per minute) to the final site of injection. The Z (depth) coordinate to reach the CA1 area in a 20–22-day-old rat is 2.6 mm depth (from height at which the cannula touches the brain). As soon as the cannula is at destination, change the flow rate to 0.1 $\mu\text{L}/\text{min}$. Inject at total of 0.5 μL of viral solution per site of injection.

During the lowering of the cannula, it is important to monitor the flow of the viral solution. We have often noticed that the flow of the viral solution is greatly reduced or stops during the lowering of the cannula and resumes suddenly at the site of injection with a gush damaging the surrounding tissue. If that is the case, we suggest first lowering the cannula at 0.5 mm into the brain while injecting sterile PBS (see point g above) to avoid infection elsewhere in the brain and wait at this height until enough pressure has built in the pump to resume the flow. Then, slowly lower the cannula to the final destination. This trick ensures that you do not damage the area to be injected by the sudden gush of viral solution, which often occurs when the flow of solution resumes.

- (l) Remove cannula slowly (0.5 mm per minute).
- (m) If both hemispheres are to be injected, repeat the procedure from point h–l.
- (n) Remove rat from frame and close skin with suture. For post-operative pain, inject the rat subcutaneously with buprenorphin solution (0.03 mg/kg).

4 Use of the Sindbis Viral Expression System to Investigate the Role of CREB in Neuronal Plasticity and Memory Formation

Figure 3 shows successful expression of a GFP-expressing Sindbis virus in the CA1 area and dentate gyrus of a young adult rat hippocampus 24 h after *in vivo* infection. Note that the virus preferentially infects the neurons of the CA1 pyramidal layer or the dentate gyrus (DG). By expressing GFP along with the protein-of-interest, we can easily identify cells that have been infected in living tissue using simple epifluorescent light microscopy and perform electrophysiological and morphological analyses. Also, providing that we infect a sufficient area of the hippocampus, we can couple this molecular manipulation to behavioral analysis. We have successfully used these combinations of techniques to evaluate the role of CREB in neuronal plasticity and memory formation. In these experiments, we have always included a control group, which consisted of infection of the sindbis virus expressing only IRES-GFP, to determine if the phenotypic alterations that we observe are due to overexpression of our protein-of-interest or due to viral infection *per se*. We have not observed significant differences

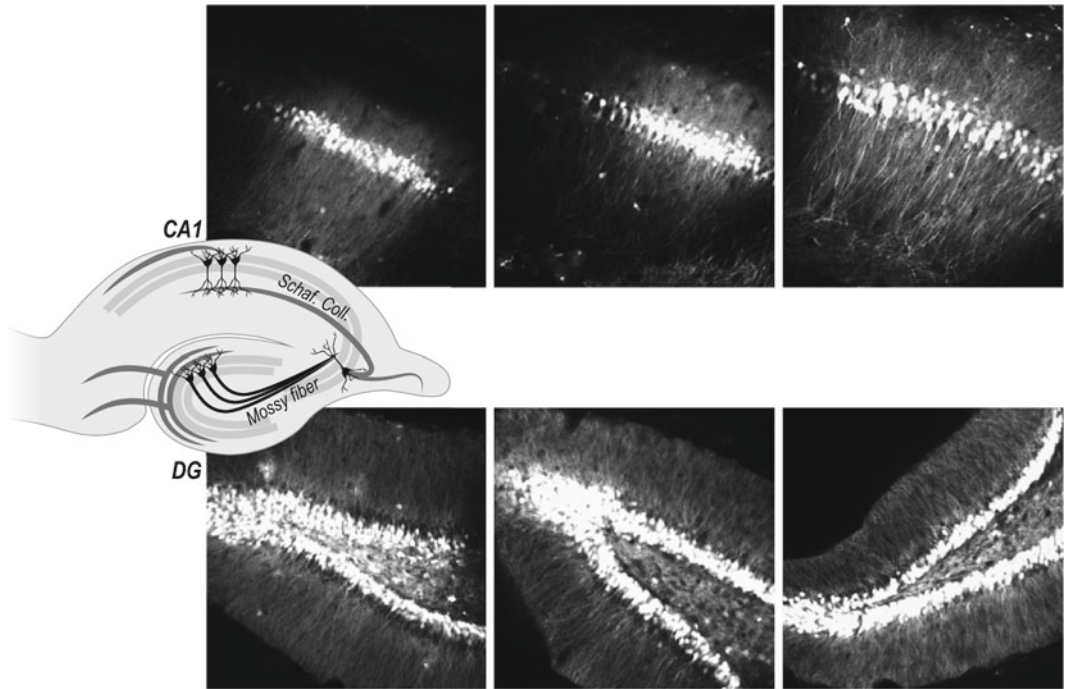


Fig. 3 Successful expression of GFP-expressing sindbis virus in the CA1 pyramidal neurons (*top*) or dentate gyrus granule cells (*bottom*) in a 21-day-old Sprague–Dawley rat 24 h after in vivo infection

between uninfected and GFP infected neurons in the electrophysiological and morphological analyses that we have performed, arguing against any cytotoxic effects of this virus within the time frame of these electrophysiological and morphological experiments (up to 48 h)[12, 13].

4.1 Role of CREB in Neuronal Plasticity

We have evaluated the role of CREB in the regulation of synaptic plasticity, intrinsic excitability, and spine formation. Upstream of the IRES-GFP, we have cloned a constitutively active form of CREB (CREBCA [14]) or a dominant negative form of CREB where serine-133 is mutated to an alanine (CREBDN [15]) [12, 13]. We were able to compare the detailed electrophysiological properties of infected vs. uninfected neighboring neurons in acutely dissected hippocampal slices from infected animals. The infected neurons showed healthy and stable synaptic transmission comparable to that recorded from uninfected neurons in the same slice preparation and also comparable to neurons from uninfected animals. We could maintain recordings for times sufficient to examine long-term potentiation (LTP) and long-term depression (LTD), and also performed many difficult electrophysiological assays such as those that required minimal stimulation techniques [13, 16]. Because standard acute hippocampal slices were used, recordings did not suffer from the drawbacks which often

accompany the use of organotypic brain slices, such as epileptiform activity and small, unstable responses. Using the *in vivo* infection approach coupled to *in vitro* electrophysiology, we demonstrated that increasing CREB-dependent transcription (by expression of CREBCA) in CA1 pyramidal neurons results in the formation of new silent synapses and increases the magnitude of LTP that can be obtained in these neurons [13]. Using the same approach in a different structure, the nucleus accumbens, we also demonstrated that modifying CREB activity bidirectionally regulates intrinsic excitability, with expression of CREBCA increasing spike frequency while expression of CREBDN decreased spike frequency [12]. The CREB-dependent increases in LTP magnitude and bidirectional regulation of spike frequency were also observed in hippocampal neurons of transgenic mice expressing another type of constitutively active CREB or dominant negative CREB [17–19], confirming the accuracy of our findings using the viral approach.

Our *in vivo* viral approach also allowed us to study the effects of *in vivo* molecular manipulations of CREB activity on the morphology of neurons. By adding a fluorescent dye (e.g., Alexa 568 fluor hydrazide from Molecular Probes) to the whole-cell pipette recording solution, we were able to fill infected cells with the dye during electrophysiological recordings. After fixing and mounting the tissue, we could visualize the detailed morphology of the infected neurons using a confocal microscope. By collecting Z-stacks of parts of apical secondary dendrites and reconstructing these in 3-D using Volocity software (Improvision), we could compare the density and morphology of dendritic spines between infected and uninfected neurons [13]. We thus demonstrated that increasing CREB function in CA1 pyramidal neurons, by expression of CREBCA, increases spine density on apical dendrites. Addition of a fluorescent dye was necessary because morphological analysis of the infected neurons using the viral GFP expression was generally not strong enough for detailed morphological analysis. Alternatively, a GFP-antibody amplification immunohistochemical procedure could in theory be used for optimized visualization of the infected neurons to perform morphological analysis.

4.2 Role of CREB in Memory Formation

We have also coupled this *in vivo* molecular manipulation to behavioral analysis to ask how increasing CREB function in the hippocampus affects hippocampus-dependent memory formation. Using this combined approach, we could accurately restrict transgene expression in a spatially and temporally restricted manner, a control that is highly valuable while evaluating the role of a protein in behavior and that cannot be easily achieved by murine genetic manipulations. We injected the CREBCA virus or the control GFP virus bilaterally in the CA1 pyramidal layer or the dentate gyrus of adult mice. One day after this infection, we trained the mice in a contextual fear conditioning task and evaluated how increasing

CREB function influenced this conditioning. We tested memory formation 24 h after training. At the end of the behavioral task, the brains were systematically fixed, sliced, and mounted to monitor accurate bilateral infection and extent of infection by GFP fluorescence. Only mice exhibiting bilateral infection to the targeted area were considered for analysis. We demonstrated that increasing CREB-dependent transcription in CA1 neurons or in DG granule cells strengthens memory formation in this task [11].

5 Conclusions

In this chapter, we described in detail the use of a sindbis viral-based gene expression system that we have optimized to express heterologous proteins in the rodent brain *in vivo*. We believe that this technique offers several advantages over other approaches that are used to express recombinant proteins *in vivo*. In particular, it allows evaluation of the effect of rapid expression of any protein-of-interest in a temporally and spatially restricted manner while minimizing the possibility of time-dependent compensations in response to the molecular manipulation. This feature is particularly relevant for combination of this molecular manipulation with behavioral analysis. It also permits direct comparison of genetically transfected and neighboring control neurons within the same tissue. Although, in this chapter, we focused on altering CREB function *in vivo*, this molecular manipulation could be adapted in theory to overexpression of any protein-of-interest, in any region of the brain at any postnatal age, thus demonstrating the very versatile and powerful nature of this technique.

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References

1. Johannessen M, Delghandi MP, Moens U (2004) What turns CREB on? *Cell Signal* 16: 1211–1227
2. Lonze BE, Ginty DD (2002) Function and regulation of CREB family transcription factors in the nervous system. *Neuron* 35:605–623
3. Hardingham GE, Fukunaga Y, Bading H (2002) Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. *Nat Neurosci* 5:405–414
4. Benito E, Barco A (2010) CREB's control of intrinsic and synaptic plasticity: implications for CREB-dependent memory models. *Trends Neurosci* 33:230–240
5. Josselyn SA, Nguyen PV (2005) CREB, synapses and memory disorders: past progress and future challenges. *Curr Drug Targets CNS Neurol Disord* 4:481–497
6. Sakamoto K, Karelina K, Obrietan K (2011) CREB: a multifaceted regulator of neuronal

- plasticity and protection. *J Neurochem* 116: 1–9
7. Kim J, Dittgen T, Nimmerjahn A et al (2004) Sindbis vector SINrep(nsP2S726): a tool for rapid heterologous expression with attenuated cytotoxicity in neurons. *J Neurosci Methods* 133:81–90
 8. Ehrenguber MU, Lundstrom K, Schweitzer C et al (1999) Recombinant Semliki Forest virus and Sindbis virus efficiently infect neurons in hippocampal slice cultures. *Proc Natl Acad Sci USA* 96:7041–7046
 9. Lundstrom K (2005) Biology and application of alphaviruses in gene therapy. *Gene Ther* 12(suppl 1):S92–S97
 10. Vetere G, Marchetti C, Benevento M et al (2011) Viral-mediated expression of a constitutively active form of CREB in the dentate gyrus does not induce abnormally enduring fear memory. *Behav Brain Res* 222:394–396
 11. Restivo L, Tafi E, Ammassari-Teule M, Marie H (2009) Viral-mediated expression of a constitutively active form of CREB in hippocampal neurons increases memory. *Hippocampus* 19:228–234
 12. Dong Y, Green T, Saal D et al (2006) CREB modulates excitability of nucleus accumbens neurons. *Nat Neurosci* 9:475–477
 13. Marie H, Morishita W, Yu X, Calakos N, Malenka RC (2005) Generation of silent synapses by acute in vivo expression of CaMKIV and CREB. *Neuron* 45:741–752
 14. Du K, Asahara H, Jhala US, Wagner BL, Montminy M (2000) Characterization of a CREB gain-of-function mutant with constitutive transcriptional activity in vivo. *Mol Cell Biol* 20:4320–4327
 15. Gonzalez GA, Montminy MR (1989) Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* 59:675–680
 16. Isaac JT, Hjelmstad GO, Nicoll RA, Malenka RC (1996) Long-term potentiation at single fiber inputs to hippocampal CA1 pyramidal cells. *Proc Natl Acad Sci USA* 93:8710–8715
 17. Barco A, Alarcon JM, Kandel ER (2002) Expression of constitutively active CREB protein facilitates the late phase of long-term potentiation by enhancing synaptic capture. *Cell* 108:689–703
 18. Jancic D, Lopez DA, Valor LM, Olivares R, Barco A (2009) Inhibition of cAMP response element-binding protein reduces neuronal excitability and plasticity, and triggers neurodegeneration. *Cereb Cortex* 19:2535–2547
 19. Lopez de Armentia M, Jancic D, Olivares R et al (2007) cAMP response element-binding protein-mediated gene expression increases the intrinsic excitability of CA1 pyramidal neurons. *J Neurosci* 27:13909–13918

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