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A rat model of EcoHIV brain infection

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TITLE

A rat model of EcoHIV brain infection

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KEYWORDS

EcoHIV; HIV; Microglia; HAND; rat

SUMMARY

Here, we present a protocol to establish a new rat model of active HIV infection using chimeric HIV (EcoHIV), which is critical for enhancing our understanding of HIV-1 viral reservoirs in the brain and offering a system to study HIV-associated neurocognitive disorders and associated comorbidities (i.e., drug abuse).

ABSTRACT

It has been well studied that the EcoHIV infected mouse model is of significant utility in investigating HIV associated neurological complications. Establishment of the EcoHIV infected rat model for studies of drug abuse and neurocognitive disorders, would be beneficial in the study of neuroHIV and HIV-1 associated neurocognitive disorders (HAND). In the present study, we demonstrate the successful creation of a rat model of active HIV infection using chimeric HIV (EcoHIV). First, the lentiviral construct of EcoHIV was packaged in cultured 293 FT cells for 48 hours. Then, the conditional medium was concentrated and titered. Next, we performed bilateral stereotaxic injections of the EcoHIV-EGFP into F344/N rat brain tissue. One week after infection, EGFP fluorescence signals were detected in the infected brain tissue, indicating that EcoHIV successfully induces an active HIV infection in rats. In addition, immunostaining for the microglial cell marker, Iba1, was performed. The results indicated that microglia were the predominant cell type harboring EcoHIV. Furthermore, EcoHIV rats exhibited alterations in

temporal processing, a potential underlying neurobehavioral mechanism of HAND as well as synaptic dysfunction eight weeks after infection. Collectively, the present study extends the EcoHIV model of HIV-1 infection to the rat offering a valuable biological system to study HIV-1 viral reservoirs in the brain as well as HAND and associated comorbidities such as drug abuse.

INTRODUCTION

Biological systems have enhanced our understanding of HIV-1 associated neurocognitive disorders (HAND) and their underlying neural mechanisms[2]. Determining which biological system is most appropriate for any given study is often dependent upon the question of interest[2]. The limitation of the range of host animal models challenges studies of HIV-1 disease development. To investigate HIV-1 viral replication and pathogenesis, Potash et al.[3] created a mouse model of active HIV-1 infection, replacing the coding region of HIV surface envelope glycoprotein, gp120, with ecotropic MLV gp80, which led to successful viral replication in mice[4]. After tail vein injections in chimeric HIV (EcoHIV) mice, many characteristics were observed resembling those of HIV-1 seropositive individuals (e.g., infected lymphocytes and macrophages, targeted for antiviral immune responses, and inflammation[3,5,6]).

Although mice and rats are both members of the Muridae, fundamental species differences may influence their suitability for specific experimental questions[7]. Therefore, the extension of the EcoHIV infection model to rats (commonly used in studies of drug abuse and neurocognitive disorders) would be advantageous in the study of neuroHIV. For example, their larger size makes jugular catheter implantation for drug self-administration procedures more practical[8]. Drug self-administration techniques in rats have been utilized to evaluate motivation in HIV-1[9]. Furthermore, many neurocognitive/behavioral tasks were initially designed for rats[10]. Here, we report the utilization of stereotaxic injections of EcoHIV in rats to extend the EcoHIV infection model and afford a key opportunity to address novel questions related to neuroHIV and HAND.

PROTOCOL

All animal protocols were reviewed and approved by the Animal Care and Use Committee at the University of South Carolina (federal assurance number: D16-00028). Six adult male F344/N rat was pair housed in a controlled environment under a 12/12 light: dark cycle with ad libitum access to food and water. All animals were cared for using guidelines established by the National Institutes of Health in the Guide for the Care and Use of Laboratory Animals.

1. Virus packaging in 293 FT cells

1.1. Culture the 293 FT cells (5×10^5/mL) in gelatin coated 75 cm^2 flasks with DMEM plus 10% FBS[11]. Grow cells at 37 °C to be 50% confluent at transfection.

1.2. Dilute 22.5 µL of transfection reagent (e.g., Lipofectamine 3000) in 750 µL of medium (e.g., Opti-MEM) in a 1.5 mL micro-centrifuge tube and vortex for 3 s.

1.3. Dilute 20 µg of EcoHIV plasmid DNA in 750 µL of medium in a 1.5 mL micro-centrifuge tube and mix well.

1.4. Add diluted DNA into the tube of diluted transfection reagent and mix gently. Incubate for 15 min at room temperature.

1.5. Add the mixture to 10 mL of prewarmed DMEM medium in 75 cm^2 flask. Incubate cells for 2 days at 37 °C.

1.6. Harvest and combine 24 mL of conditional medium from the two flasks that include the packaged lentivirus.

1.7. Centrifuge all 24 mL of conditional medium at 500 x *g* for 10 minutes at 4 °C. Transfer the clarified supernatant to a sterile 50 mL tube.

1.8. Combine 8 mL of Lenti-x concentrator with 24 mL of clarified supernatant (1:3 ratio). Mix by gentle inversion. Incubate mixture at 4 °C for two days.

1.9. Centrifuge mixture at 1,500 x *g* for 45 minutes at 4 °C. Carefully remove the supernatant.

1.10. Gently re-suspend the pellet with 100 µL of 100 mM PBS.

1.11. Titer virus concentration with a p24 ELISA kit.

2. EcoHIV-EGFP virus stereotaxic surgeries

2.1. Anesthetize rats using 3% sevoflurane. Proceed to step 2.2 when the rats are not responsive to noxious stimuli and reflexes are absent.

2.2. Shave the hairs from the brain region and sterilize the skin twice with 70% ethanol. Secure the rat in a prone position in the stereotaxic apparatus.

2.3. Make an incision (5-6 cm) through the skin along the scalp midline.

2.4. Mark two drilling positions at 0.8 mm lateral, 1.2 mm rostral to bregma. Drill a hole (diameter 0.4 mm) in each skull position.

2.5. Fill titered EcoHIV lentivirus solution (1.04×10^6 TU/mL) in a 10 µL injection syringe. Secure the syringe to the stereotaxic apparatus.

2.6. Move down the needle close to the surface of drilling hole. Measure and move 2.5 mm in depth.

2.7. Infuse 1 µL of virus solution at a rate of 0.2 µL/min. Keep the needle inside of the injection area for 5 min. Slowly move the needle up until it is outside of the rat skull.

2.8. Suture the skin with a 4-0 silk thread.

2.9. Sterilize the incision with 70% ethanol once. Subcutaneously inject butorphenol (Dorolex, 0.1 mg/kg body weight).

2.10. Transfer the rat to a recovery chamber with a heating pad until it wakes up.

3. Visualization of brain sections

NOTE: Wait one to eight weeks after EcoHIV viral infusion.

3.1. Anesthetize the rat with 5% sevoflurane. Continue to step 3.2 when the rats are not responsive to noxious stimuli and reflexes are absent.

3.2. Fix the rat in a supine position inside a fume hood.

3.3. Incise the skin along the thoracic midline. Cut the diaphragm and open the thoracic cavity.

3.4. Insert a 20 G × 25 mm needle into the left ventricle.

3.5. Immediately open the right atrium with scissors.

3.6. Perfuse 50 mL of prechilled 100 mM PBS at a rate of 5 mL/min.

3.7. Perfuse 100 mL of cold 4% paraformaldehyde at a rate of 5 mL/min.

3.8. Decapitate the rat, open the scalp and remove the brain.

3.9. Postfix overnight with 4% paraformaldehyde.

3.10. Transfer the brain to 40 mL of 30% sucrose in 100 mM PBS in 50 mL tube until the brain floats down to the bottom (about 3 days).

3.11. Snap freeze the brain in methylbutanol for 2 min at – 80 °C.

3.12. Secure the brain tissue on a metal platform inside a -20 °C cryostat.

3.13. Cut 50 µm thick coronal sections using the cryostat.

3.14. Transfer the brain slices onto glass slides with a fine brush.

3.15. Mount sections in 0.3 mL of antifade medium and cover with 22 mm × 50 mm coverslips.

3.16. Keep the glass slides in the dark at room temperature till dry.

3.17. Image the targeted neurons with a confocal microscope using Z-stack based on brain region boundaries and morphological characteristics of neurons.

NOTE: The confocal microscope settings used were: magnification of 60 X (A/1.4, oil), and a Z-plane interval of 0.15 µm (pinhole size 30 µm; back-projected pinhole radius 167 nm) using a wavelength of 488 nm.

REPRESENTATIVE RESULTS

The conditioned medium was collected from lentivirus of EcoHIV-EGFP infected 293FT cells. Next, it was concentrated and titered, then stereotaxically injected into the brain (cortical region) of F344/N rats. Seven days post-injection, rats were sacrificed and images were taken from coronal brain slices ranging from bregma 5.64 mm to bregma -4.68 mm. In **Figure 1A**, there are significant EcoHIV-EGFP signals throughout the brain, especially in the cortex and the hippocampal dentate gyrus. Furthermore, dual-labeling with Iba1and EcoHIV-EGFP probes provided strong evidence that microglia were the predominant cell type harboring EcoHIV expression in the brain (**Figure 1B**). Notably, the distribution pattern of EcoHIV-EGFP is consistent with the relative regional concentrations of microglia in the rat brain (i.e., cortex and dentate gyrus of the hippocampus).

In a subsequent study, we validated the utility of EcoHIV infection in rats to model key aspects of HAND. Using the protocol detailed above, F344/N rats were stereotaxically injected with either EcoHIV-EGFP or saline. First, eight weeks post-infection, temporal processing, a potential elemental dimension of HAND[12], was evaluated using visual gap prepulse inhibition (**Figure 2**). EcoHIV animals exhibited a relative insensitivity to the manipulation of interstimulus interval (ISI), evidenced by a relatively flatter ISI function compared to saline controls. Specifically, significant differences in the slope of the ISI function from the 50 ms ISI to the 200 ms ISI were observed (Semilog Line-X is Log, Y is Linear, R^2s ≥ 0.99; $F(1,2)=642.9$, $p \leq 0.001$). Second, ballistic labeling was used to investigate the impact of EcoHIV-EGFP injections on the morphology of dendritic spines in medium spiny neurons (MSN) of the nucleus accumbens (NAc; **Figure 3**); parameters which can be utilized to draw inferences about synaptic function[13]. EcoHIV rats displayed profound alterations in dendritic spine morphology, evidenced by an increased relative frequency of shorter dendritic spines (Genotype x Bin Interaction, $F(16, 218) = 4.3$, $p \leq 0.001$) with an increased head diameter (Genotype x Bin Interaction, $F(12, 96) = 18.7$, $p \leq 0.001$) and increased neck diameter (Genotype x Bin Interaction, $F(15, 120) = 16.3$, $p \leq 0.001$) relative to control animals. Detailed methodology for the assessment of temporal processing[14] and ballistic labeling[13] have been previously reported.

Figure Legends

Figure 1. **The EcoHIV-EGFP infected cells distributed in rat brain.** (**A**) The representative confocal images (20x) of EcoHIV-EGFP expression in hippocampal dentate gyrus or cortex regions at 7 days after injection. (B) The representative confocal images (60X) of co-localization of Iba1 immunostaining with EcoHIV-EGFP infected cells at 7 days after injection.

Figure 2. **EcoHIV infection induced prominent neurocognitive deficits in temporal processing.** Visual gap prepulse inhibition was conducted eight weeks after stereotaxic injections of either EcoHIV or saline. EcoHIV infection induced prominent alterations in temporal processing evidenced by the relative insensitivity to the manipulation of interstimulus interval relative to control rats. Detailed methodology described in McLaurin et al.[13].

Figure 3. **Infectivity with EcoHIV-EGFP altered the morphological parameters of dendritic spines, supporting profound synaptic dysfunction.** EcoHIV rats displayed profound alterations in dendritic spine morphology, evidenced by an increased relative frequency of shorter dendritic spines (**A**) with an increased head diameter (**B**) and increased neck diameter (**C**) relative to control animals.

DISCUSSION

In this protocol, we established an EcoHIV-induced HIV infection model in rats. Specifically, we described a bilateral stereotaxic injection of EcoHIV into the cortex which successfully induced active HIV infection in the rat brain 7 days post-injection. Futhermore, we demonstrate that EcoHIV infection in rats could be a good biological system to study key aspects of HAND. Eight weeks post- EcoHIV infection, rats exhibited significant neurocognitive impairments, which included the alterations in temporal processing and synaptic dysfunction in MSNs of the NAc. Given the importance of animal models for the study of neuroHIV and HAND[2], the development of a new biological system may be advantageous for addressing novel questions within the field. Potash et al.[3] first reported the use of EcoHIV to induce active HIV-1 infection in mice. Specifically, mice were inoculated with a 0.1 mL of solution of EcoHIV virus through tail vein injections[3]. Six weeks after infection, the HIV-1 viral DNA was detected in spleen cells and lymphocytes. Additionally, one inoculation of EcoHIV injection was sufficient to induce infection in more than 75% of the mice. Utilization of bilateral stereotaxic injections, as in this study, successfully infected 100% of the rats (n = 6 or n = 4, respectively) evidenced by the detection of EcoHIV-EGFP in the brain seven days post-injection.

Previous studies have shown that infection by EcoHIV in the mouse strongly implicated that brain microglia are highly susceptible to EcoHIV virus infection[3]. In this study, combined with Iba1 (a microglial cell marker) immunostaining, a significant co-localization of EGFP signal with Iba1+ cells was observed, strongly suggesting that microglia were the major cell type for EcoHIV infection in rat brain. Observations of significant EcoHIV infection in microglia are consistent with data in EcoHIV infected mice[6], as well as HIV-1 seropositive individuals[15] and other biological systems commonly utilized to model HIV[16,17]. We also performed retro-orbital injection of EcoHIV-EGFP into F344/N rats and the data also indicated high expression of EcoHIV-EGFP in both the cortex and hippocampal dentate gyrus after only seven days (data not

shown). In contrast, I.P. injection of EcoHIV in F344/N rats led to undetectable viral expression in the rat brain, inspite of high doses of EcoHIV lentivirus.

Regarding this protocol, researchers should ensure that the conditioned medium, including the EcoHIV lentivirus packaging in 293 FT cells, is concentrated and titered before using for stereotaxic injection. These steps are critical to ensuring consistent and replicable results across experiments. Furthermore, we also found that EcoHIV infection was propagated from stereotaxic injection in brain to spleen tissue at 8 weeks after injection. Meanwhile, the temporal processing deficits were observed in EcoHIV-infected rats as early as 14 days and maintained through 8 weeks post-infection (the current experimental terminal time, **Figure 2**). Compared to the generalized EcoHIV infection model in mice, the more specific regional stereotaxic injection of EcoHIV produced efficient HIV infection in brain areas and produced a temporal processing deficit in rats, which is key for the study of HIV associated neurocognitive dysfunction.

Limitations providing further opportunities for the current protocol include the identification of other cell types such as neurons and astrocytes, a time-course study of changes in EcoHIV-EGFP expression after infection to indicate viral replication and latency in brain, a longitudinal study to address the potential impacts of HIV associated neurocognitive deficits, and evaluating if the stereotaxic injection of EcoHIV evades the immune system further spreading viral expression throughout the body. Additionally, this should be tested on other rat strains to confirm the generalizability of EcoHIV infection in rats.

ACKNOWLEDGMENTS:
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DISCLOSURES:
None of the authors have conflicts of interest to declare.

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Figure 1

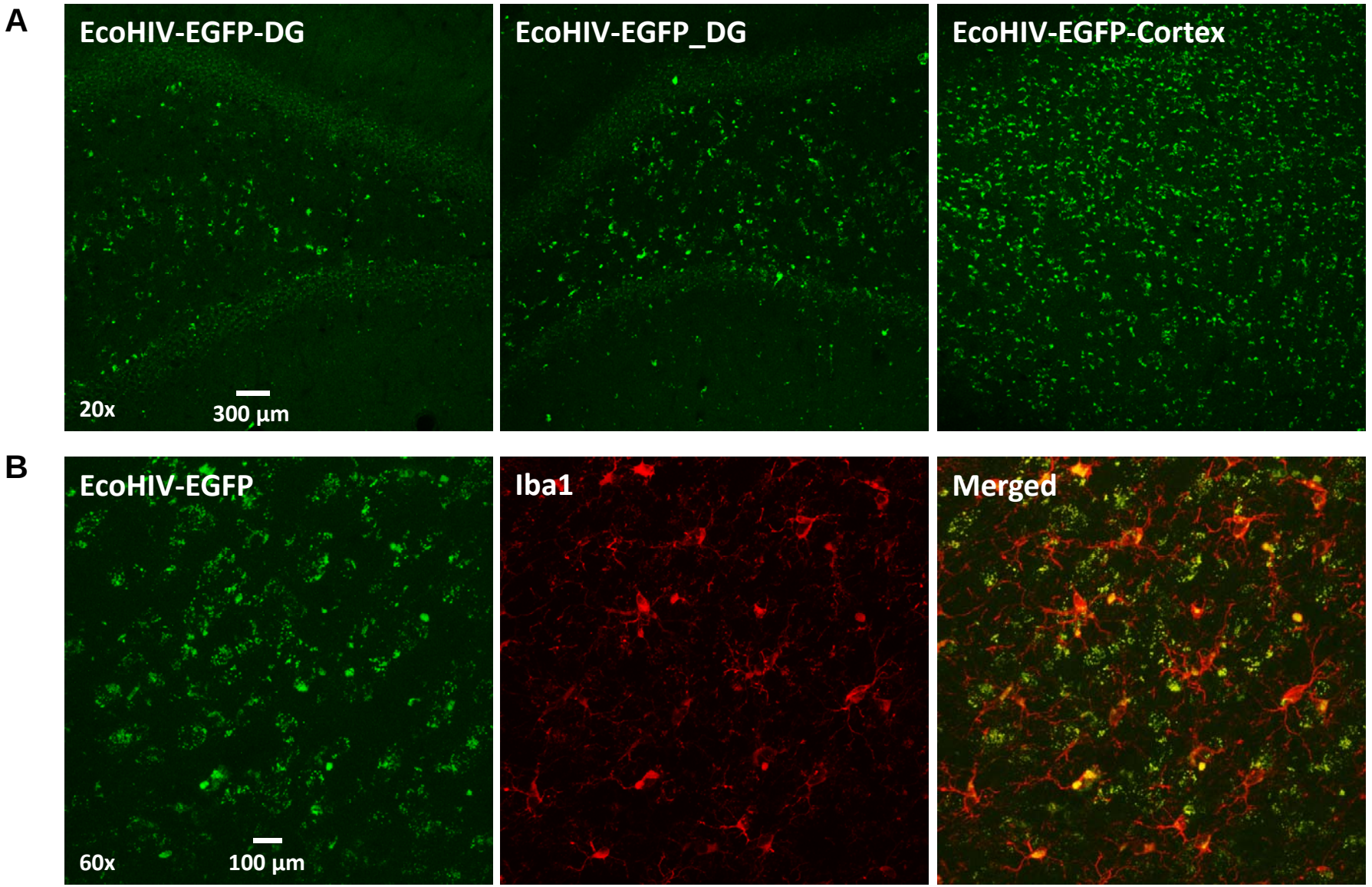

Figure 2

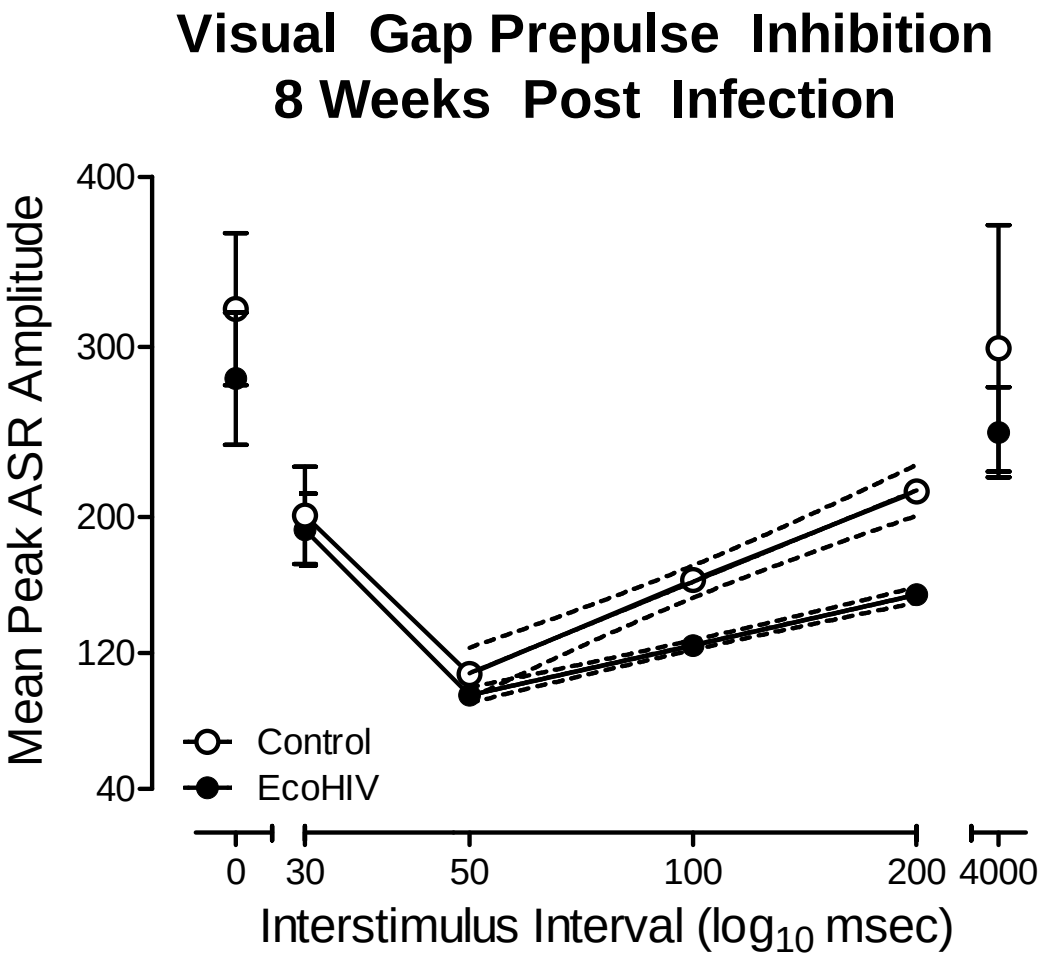

Figure 3

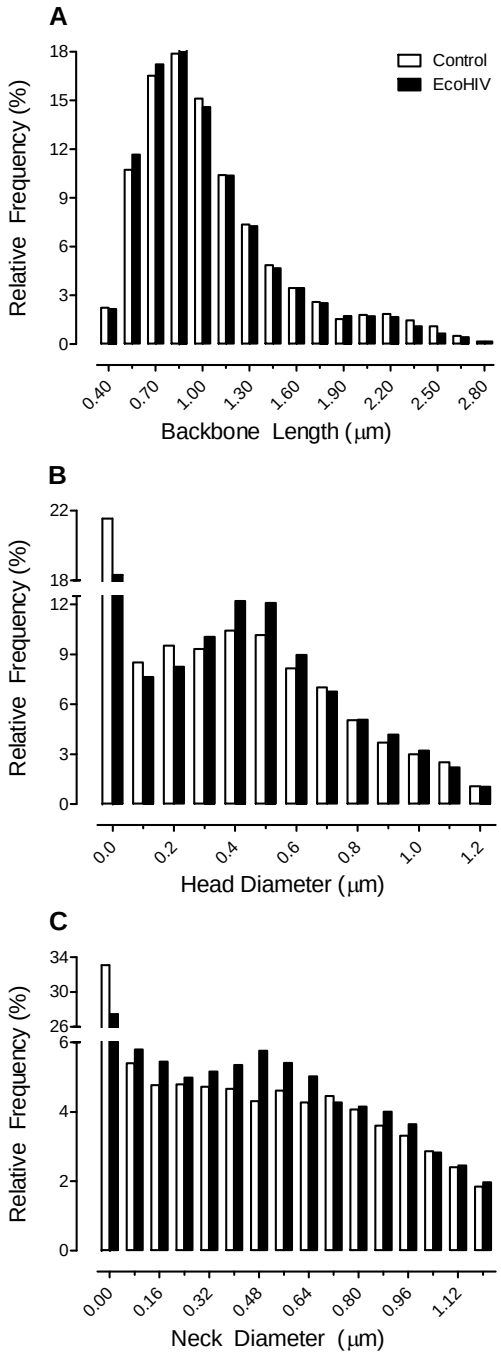

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
293FT cells	ThermoFisher Scientific	R70007	
Antibiotic-Antimycotic solution	Cellgro	30004CI	100X
Corning BioCoatGelatin 75cm² Rectangular Canted Neck Cell Culture Flask with Vented Cap	Life Technologies	354488	
Corning DMEM with L-Glutamine, 4.5 g/L Glucose and Sodium Pyruvate	Life Technologies	10013CV	
Cover glass	VWR	637-137	
drill			
Dumont #5 Forceps	World Precision Instruments	14095	
Dumont #7 Forceps	World Precision Instruments	14097	
Eppendorf Snap-Cap Microcentrifuge Biopur Safe-Lock Tubes	Life Technologies	22600028	
Ethicon Vicryl Plus Antibacterial, 4-0 Polyglactin 910 Suture, 27in. FS-2	Med Vet International	VCP422H	
Hamilton syringe	Hamilton	1701	
Invitrogen Lipofectamine 3000 Transfection Reagent	Life Technologies	L3000015	
Iris Forceps	World Precision Instruments	15914	
Iris Scissors	World Precision Instruments	500216	
Lentivirus-Associated p24 ELISA Kit	Cell Biolabs, inc.	VPK-107-5	
Lenti-X Concentrator	Takara	PT4421-2	
Opti-MEM I Reduced Serum Medium	Life Technologies	11058021	
Paraformaldehyde	Sigma-Aldrich	158127-500G	
Paraformaldehyde	Sigma	P6148	
ProLong Gold	Fisher Scientific	P36930	
Sevoflurane	Merritt Veterinary Supply	347075	
stereotaxic apparatus	Kopf Instruments	Model 900	
SuperFrost Plus Slides	Fisher Scientific	12-550-154%	
Vannas Scissors	World Precision Instruments	500086	

Dec 16, 2020

Dr. Vineeta Bajaj,
Review Editor
Journal of Visualized Experiments
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Cambridge, MA 02140

Dear Dr. Vineeta Bajaj,
Thank you very much for your kind consideration of our manuscript entitled "A rat model of EcoHIV-induced HIV infection" by Dr. Li and co-authors for consideration for publication in *JoVE*. We greatly appreciated your suggestions regarding the revision. We also revised the manuscript according to the suggestions provided by the reviewers and reorganized the procedure and discussion sections.
We are now resubmitting this revised manuscript to *JoVE*. Please feel free to let us know if you have any questions.
Sincerely,

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Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Answer: It has been checked.

2. Please ensure that corresponding reference numbers appear as numbered superscripts after the appropriate statement(s) for in-text formatting
Answer: It has been revised.

3. Please revise the following lines to avoid overlap with previously published work: 37-40, 46-48, 170-189, 202-218, 223-230, 253-258, etc.
Answer: It has been rewritten.

4. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
Answer: It has been done.

5. Line 90: Please explain how you culture the 293 FT cells. The number of cells used for seeding. How do you coat gelatin? What percentage of gelatin is used and how long is flask incubated to ensure gelatin-coating? What is the culture-medium used?
Answer: It has been rewritten.

6. Line 104: Do you remove the medium before adding the transfection mix? Do you remove the transfection mix and add fresh medium before harvesting the conditional medium?
Answer: It has been done.

7. Line 111: Please include the details of what is centrifuged (i.e., conditioned medium in the centrifuge tube).
Answer: It has been done.

8. Line 140: Please mention how big is the incision.
Answer: The info has been added.

9. Line 146: What is the MOI/concentration of lentivirus solution?
Answer: It has been added.

10. Line 164: Do you provide any analgesics to the animal?
Answer: It has been added.

11. Line 185 and 187: Please mention the rate of perfusion.
Answer: It has been added.

12. Line 189: Please mention the steps in the removal of the rat brain.
Answer: It has been mentioned.

13. Line 208: Please mention the temperature at which the glass slides are stored.
Answer: The info has been added.

14. In the discussion section please also include some limitations of the protocol as well.
Answer: A new paragraph has been added to describe the limitations of the current protocol.

15. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text. Please ensure that each Figure Legend includes a title and a short description of the data presented in the Figure and relevant symbols.
Answer: It has been done.

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17. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.
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18. Please upload each Figure individually to your Editorial Manager account.
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19. Figure 2: Please define the terms used in the X and Y- axis in the Figure Legends section. Please include the details of the statistics used.
Answer: It has been revised.

20. Please remove trademark (™) and registered (®) symbols from the Table of Materials. Please revise the table of materials in alphabetical order.
Answer: It has been done.

_____

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
This is an admirably clear methods paper. It is likely to be valuable as a descriptive protocol for infection of brain in living rats by chimeric HIV and subsequent study of HAND, in particular by behavioral changes.

Major Concerns:
none

Minor Concerns:
on lines 269-70 the authors state ...."peritoneal macrophages in the mouse are infected through pathogenic viral proteins, such as Tat".... The protein Tat does not "infect". The intended meaning is unclear and the manuscript would benefit from rephrasing this thought.
Answer: It has been rewritten.

Reviewer #2:

Manuscript Summary:
This protocol described the generation of rat HAND model using ecoHIV infection in the brain. As compared with mouse model, rat models are frequently used for studies on drug abuse and neurocognitive impairment, with clear advantages over mouse model. The topic is interesting and significant.

Major Concerns:

1). The rationale to use the brain injection was not justified clearly. Both I.P. and I.V. injections have been demonstrated for nice mouse ecoHIV modeling, that also involve neurocognitive abnormalities. For specific brain cell susceptibility (particularly microglia infection and latency), local injection is a good selection.
Answer: In previous studies, it was reported that about 75% of experimental mice successfully established HIV infection after EcoHIV inoculation through tail vein. However, our method of bilateral stereotaxic injection could produce 100% infection efficiency. Plus, it turns out that microglia are more sensitive and serve as the predominant cell type in brain after EcoHIV infection regardless of the route of administration. Anecdotally, in our study, the retro-orbital injection of EcoHIV-EGFP to F344/N rat also elicited high expression of EcoHIV-EGFP in both the cortex and hippocampal dentate gyrus after only seven days. However, I.P. injection of EcoHIV in F344/N rat not only need high dose of EcoHIV lentivirus, but also show undetectable viral expression in rat brain from our data.

2). All the three procedures: ecoHIV (lentivirus) packaging, stereostaxic injection and perfusion fixation, are very well established, thus nothing important has been added. In the manuscript, ecoHIV-EGFP

reporter virus was used and the EGFP expression pattern and dynamic changes were nicely described. However, the dose-response (different titers used and various extents of behavioral changes) was not presented. The value and data for the temporal processing (Fig 2) and ballistic labeling (Fig 3) were discussed and presented but not in the protocol, although both have been published in JOVE (the described three procedures have been also widely published). The stereostatic injection region was not stated clearly. Different regions such as hippocampus, striatum, hypothalamus, and cortex might provide more interesting protocols.

Answer: We truly understand that parts of the methodologies have been described in the field. However, this is the first report about a rat model of EcoHIV infection. This extension of EcoHIV infection model to rats would be beneficial for studying the cognitive and behavioral dysfunctions, especially for HIV associated neurocognitive disorders.
Meanwhile, our previous publications demonstrated that HIV-1 transgenic rats show temporal processing deficits due to injuries of the pyramidal neurons in rat prefrontal cortex and media spiny neurons in nuclear accumbens. Therefore, in this methodology, we prefer to stereotaxic inject EcoHIV virus close to the mentioned region. This will allow us to compare EcoHIV brain infection with the traditional HIV-1 transgenic rats.

3). In Fig. 1, the EGFP cells look strange: everywhere? absence in cell processes(fibers). Immunostaining with anti-EGFP antibody should be used to validate the expression and increase the detection sensitivity. In addition to microglia detection, other cell types need identified.
Answer: Thank you for the suggestion. Actually, we also performed astrocyte and neuron cell markers' immunostaining. The results showed that microglia are absolute predominant cell type harboring EcoHIV in brain.
The images in Fig. 1 are located at cortex and dentate gyrus of hippocampus 7 days after EcoHIV infection, and those are the two major microglia regions in brain. Considered the high co-localization of microglia and EcoHIV expression, the EGFP signals show high density.

4). The dynamic changes in EGFP cell number and cellular pattern at different time after infection would provide interesting insight at the virus replication and expansion or latency in the brain.
Answer: Thank you for the suggestion. We already have those data in another study which is under revision now.

Minor Concerns:
The title does not reflect the HAND modeling. Also, the "ecoHIV-induced HIV infection" looks abundant wording. Suggested title: A rat model of ecoHIV brain infection.
Answer: Thank you for the suggestion and it has been changed.