Intrastriatal delivery of integration-deficient lentiviral vectors in a rat model of Parkinson's disease

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Summary

Standard integration-proficient lentiviral vectors (IPLVs) are effective at much lower doses than other vector systems and have shown promise in several gene therapy approaches. Their main drawback is the potential risk of insertional mutagenesis. Novel biosafety-enhanced integration-deficient lentiviral vectors (IDLVs) offer a significant improvement and comparable transduction efficacy to their integrating counterparts in some central nervous system applications. We describe here methods for (1) production of IDLVs (and IPLVs), (2) IDLV/IPLV delivery into the striatum of a rat model of Parkinson's disease, and (3) *post-mortem* brain processing.

Keywords: Integration-deficient lentiviral vectors, 6-OHDA, intrastriatal injection, Parkinson's disease

1. Introduction

Gene therapy approaches have offered promise for many disorders, and lentiviral vectors (LVs) are one of the most attractive viral vector-based systems. LVs have many positive features derived from the biology of the corresponding natural viruses and their extensive vector development ***(1-3)***. In particular, LVs can transduce a variety of cell types of the central nervous system (CNS), including dividing as well as non-dividing cells, with stable long-term expression of the transgene ***(4, 5)***. However, a potential obstacle for the routine clinical use of current integration-proficient LVs (IPLVs) is the risk of insertional mutagenesis caused by integration of the viral provirus into the host cell genome ***(6)***. This risk could be addressed by using integration-deficient LVs (IDLVs), without a reduction in vector transduction efficiency if the target cell population does not divide significantly ***(7-9)***. It may also be possible to use replicating IDLVs for stable expression from viral episomes in dividing cells ***(10)***.

IDLVs are commonly produced by targeted changes of individual amino acids within the catalytic active site of integrase (class I mutations), most frequently encoding a D64V change, which inhibit viral integration but leave other viral processes unaffected ***(11, 12)***. This strategy has mediated nearly complete (99%) inhibition of viral integration without significantly affecting proviral synthesis or infectious titres ***(9, 11-13)***. The failure to integrate into the host genome leads to increased levels of episomal viral DNA ***(8, 9, 14)***. These viral episomes are mostly converted into circles that lack replication signals, and are stable in quiescent cells but progressively diluted in proliferating cells ***(9)***. Hence, IDLVs are ideally suited for applications in the post-mitotic central nervous system (CNS) environment ***(9, 15, 16)***.

Very recently, we have assessed bio-safety and transduction efficiency of IDLVs in an animal model of Parkinson's disease, the 6-hydroxydopamine (6-OHDA)-lesioned rat, with IPLVs used as a reference ***(17)***. Examination of reporter gene (enhanced green fluorescent protein, *eGFP*) and therapeutic transgene (Glial cell-derived neurotrophic factor, *GDNF*) expression has shown efficient, long-lived and transcriptionally targeted expression from IDLVs in the striatum of injected rats. We have confirmed the lack of significant integration of IDLVs in injected rat brains by linear amplification-mediated PCR analysis followed by deep sequencing and insertion site analysis ***(17)***.

We regard these results as very encouraging for future IDLV-mediated gene therapy approaches. In the current chapter, we provide a detailed description of protocols to produce IDLVs (and IPLVs). We also present methods for delivering LVs into the striatum of 6-OHDA-treated rats and for *post-mortem* brain processing. We hope that the comprehensive descriptions in this chapter will promote a broader application and facilitate the study of IDLVs in the CNS.

2. Materials:

2.1. Lentiviral vector production

1. HEK293T cells
2. Culture medium: DMEM (Dulbecco's modified Eagle's medium) high glucose supplemented with 10% FBS (foetal bovine serum), 100 units/ml penicillin, 100 μg/ml streptomycin, stored at 4°C
3. 15 cm tissue culture plates
4. Tissue culture-grade water, stored at room temperature (RT)
5. 1x endotoxin-free TE (Tris/EDTA) buffer, filter-sterilised through a 0.22 μm filter, stored at RT
6. 2.5 M $CaCl_2$: dissolved in water, filter-sterilised through a 0.22 μm filter, aliquoted, and stored at -20 or -80°C
7. 2x HBS: 100 mM HEPES, 281 mM NaCl, 1.5 mM Na_2HPO_4, dissolved in water, adjusted to pH 7.12 (pH is crucial), filtered through a 0.22 μm filter, aliquoted, and stored at -20 or -80°C. Use freshly-thawed reagent only
8. 1 M $MgCl_2$: dissolved in water, filter-sterilised through 0.22 μm filter and stored at 4°C
9. DNAse I: stored at -20° C
10. Polyallomer ultracentrifuge tubes
11. Ultracentrifuge compatible with SV32-Ti rotor (Beckman Coulter, UK)

2.2. Stereotactic injection of LVs

1. Sprague-Dawley rats (250-300 g), maintained in a standard 12-hour light/dark cycle with free access to food and water. Experiments are performed in accordance with the UK Animals (Scientific Procedures) Act, 1986
2. Paxinos and Watson Rat Brain Atlas ***(18)***
3. Stereotactic frame (World Precision Instruments, UK)
4. UltraMicroPump III (World Precision Instruments, UK)
5. Ideal Micro drill (Harvard Apparatus, UK)
6. Shaver
7. Surgical tools (i.e. scalpels, scissors, tweezers, absorbable Vetsuture, Halsey needle holder)
8. 25 μl injection syringe with compatible stainless steel 33G needle (Hamilton, UK)
9. 5 ml syringes
10. 26G needles
11. 5% Emla cream (AstraZeneca, UK)
12. Aqupharm solution (0.18% sodium chloride + 4% glucose)
13. Isoflurane
14. 100% Oxygen
15. 70% ethanol
16. ddH_2O
17. Heat pads
18. Paper towels
19. Clean cages with bedding, food, and water

20. LVs, kept on ice during procedure

2.3. 6-OHDA lesioning

1. All materials listed in **Section 2.2**, except #20
2. 6-OHDA (6-Hydroxydopamine): dissolved in 0.9% sterile saline and 0.02% ascorbic acid (2.5 μg/μl). Store on ice, protect from light and use within a day
3. Pargyline (5 mg/ml) and desipramine (25 mg/ml): dissolved in sterile water and stored at RT

2.4. *Post-mortem* brain processing and immunohistochemistry staining

1. CO_2 chamber
2. 1x PBS (phosphate buffered saline): 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, dissolved in 1L water, adjusted to pH 7.4, and stored at RT
3. 4% PFA (paraformaldehyde): dissolved in 1x PBS, adjusted to pH 7.4 and preferably used within a day (or stored at -20°C)
4. Vibrating microtome (Campden Instruments, UK)
5. Paint brush
6. Blocking buffer: 1% BSA (bovine serum albumin) and 0.02% sodium azide, dissolved in 1x PBS-T (0.25% Triton X-100 in 1xPBS), and stored at 4°C
7. 1 M Tris buffer: Trizma base dissolved in water, adjusted to pH 7.8 with 1M HCl, and stored at RT
8. Sodium azide: 0.1% stock dissolved in water and stored at RT
9. Mowiol solution:
    - Stir 4.8 ml glycerol and 2.4 g Mowiol in 6 ml water for 2 h, RT

- Add 12 ml of 0.2 M Tris buffer pH 8.5, 0.02% sodium azide
- Incubate solution in 50-60^{o}C water bath for 10 min, stirring frequently
- Centrifuge at 5000 *g* for 15 min
- Collect supernatant, aliquot and store at -20^{o}C (*see* **Note** 1**)**

10. PPD (*p*-Phenylenediamine) solution: 0.1% PPD dissolved in water, protected from light and stored at -20^{o}C
11. Mounting solution: mix 1 part of PPD solution with 9 parts of Mowiol solution; maintain at RT, protect from light and use within a day
12. Antibodies (as required)
13. DAPI (4,6-Diamidino-2-phenylindole): dissolved in water, protected from light and stored at -20^{o}C
14. SuperFrost slides and coverslips

3. Methods

All *in vitro* work in **section 3.1** is carried out under sterile cell culture conditions.

3.1. Production of LVs by transient calcium phosphate transfection *(5, 9, 19)*

1. Seed HEK293T cells in 15 cm plates (*see* **Note** 2 for cell density) and culture until the cells are around 60% confluent (~$2x10^7$/plate/25 ml medium)
2. Two hours (minimum 30 min) prior to transfection of LV plasmids, replace the culture medium with 20 ml fresh medium per plate
3. Prepare mixture of plasmid DNA in 15 or 50 ml Falcon tube. The amount below is for each 15 cm culture plate, scale up/down as required (*see* **Note** 3):
    - To produce 2nd generation LVs, use a three-plasmid system at molar ratio 1:1:2 of packaging:env:transfer plasmids:
        - Packaging plasmid (pCMVΔR8.74 for IPLVs or pCMVΔR8.74intD64V for IDLVs): 16.25 μg
        - Envelope plasmid (pMD2.VSV-G): 7 μg
        - Transfer plasmid (containing the transgene of interest within pHR' lentiviral backbone): 25 μg (if the size of promotor + transgene is ≤ 1,500 bp) or 32 μg (if the size of promotor + transgene is ≤ 3,000 bp)
    - To produce 3rd generation LVs, use a four-plasmid system at molar ratio 1:1:1:2 of packaging:rev:env:transfer plasmids:
        - Packaging plasmid (pMDLg/pRRE for IPLVs or pMDLg/pRREintD64V for IDLVs): 12.5 μg
        - REV plasmid (pRSV-REV): 6.25 μg
        - Envelope plasmid (pMD2.VSV-G): 7 μg

- Transfer plasmid (containing the transgene of interest within pRRL or pCCL lentiviral backbone): 25 μg (if the size of promotor + transgene is ≤ 1,500 bp) or 32 μg (if the size of promotor + transgene is ≤ 3,000 bp)

4. Make up the DNA mix to 112.5 μl with 1x TE buffer
5. Top up with 1,012.5 μl tissue culture-grade water
6. Add 125 μl 2.5 M $CaCl_2$, vortex and leave for 5 min, RT
7. Add 1,250 μl 2x HBS drop-wise while vortexing DNA/$CaCl_2$ mix at full speed
8. Immediately add the mix to HEK293T cells and gently mix with the culture medium
9. Put cells back in incubator (maintained at 37°C, 5% CO_2)
10. Sixteen hours post-transfection, remove the medium and replace with 18 ml fresh medium per plate
11. Twenty four hours after medium change, harvest cell supernatant, which contains viral vector particles (*see* **Note** 4)
12. Centrifuge at 2,500 rpm (540 *g*) for 10 min, RT
13. Filter supernatant through a 0.22 μm Nalgene filter (or a 0.45 μm Nalgene filter to minimize vector loss)
14. Transfer filtered medium to high speed polyallomer centrifuge tubes (16 ml/tube)
15. Ultracentrifuge at 23,400 rpm (50,000 *g*) for 2 h, 4°C
16. Discard supernatant and keep tubes upside down on sterile paper towels for a few minutes to drain the remaining supernatant. Dry the last drops around the rim with paper towels
17. Add 50 μl of DMEM without supplements (or 1x PBS if preferred) per tube
18. Pipette up and down several times and transfer to 1.5 ml Eppendorf tube
19. Centrifuge for 10 min at 4,000 rpm (864 *g*), 4°C to remove any aggregates.

20. Transfer the supernatant to new Eppendorf tubes
21. Adjust vector stock to 10 mM $MgCl_2$ with 1 M $MgCl_2$
22. Add 5 units/ml DNAse I and incubate for 30 min, 37^{o} C
23. Aliquot and store at -80^{o} C
24. Titrate vector stock to standardize amount of vector injected (*see* **Note** 5)

3.2. Stereotactic injection of LVs

1. Use Paxinos and Watson Rat Brain Atlas to determine injection site(s) (*see* **Note** 6)
2. Set up stereotactic frame with nose bar at -3.3 mm (below the horizontal)
3. Set up UltraMicroPump for automatic injection rate at 0.5 μl/min
4. Autoclave all surgical tools prior to use
5. Sterilize Hamilton syringe and needle with 70% ethanol, then wash with ddH_2O and prime with viral vectors to prevent adsorption of the vector during dosing
6. Place rat into an anaesthesia chamber and induce with 5% isoflurane in 100% O_2 until the animal goes into deep anaesthesia (heart beats slowly and regularly)
7. Shave the head fur and place rat on a heat pad, within the stereotactic frame, with anaesthesia maintained using approximately 2.5% isoflurane in 100% O_2
8. Place rat into stereotaxic frame and hang incisors on incisor bar. The rat is ready for surgery following loss of pedal withdrawal reflex and eye-blink reflex. Monitor state of anaesthesia throughout surgical procedure
9. Cover two ear bars (of stereotactic frame) with Emla cream and set up the bars (*see* **Note** 7)
10. Make a longitudinal incision in the scalp starting from the mid-line between the eyes and extending for ~1 mm towards the tail

11. Keep the scalp open with tweezers
12. Gently wipe the skull with sterile paper towels
13. Determine the position of bregma (*see* **Note** 8) and calculate the final values of AP and ML according to it
14. Drill a burr-hole into the skull at the identified position and pierce dura using an sterile needle
15. Read the DV value, calculate the final value needed and move the syringe down to the position corresponding to this value (*see* **Note** 6)
16. Inject LVs using UltraMicroPump at a rate of 0.5 µl/min (*see* **Note** 6)
17. During injection, rehydrate the rat with Aqupharm solution (10 ml/kg, *s.c.*)
18. After injection, leave the needle in place for ~3 min prior to retracting it
19. Suture the scalp, place rat in a clean, warm cage and wait until the animal regains consciousness
20. Clean Hamilton needle and Vetsuture with 70% ethanol for the next injection
21. After the last injection clean Hamilton syringe with 70% ethanol, then water before returning to its container

3.3. 6-OHDA lesioning

1. Thirty minutes prior to surgery, inject rat with a combined solution of pargyline (5 mg/kg, i.p) and desipramine (25 mg/kg, i.p) (*see* **Note** 9)
2. Carry out all steps listed in **Section 3.2**. However,

   - In step 5: prime the syringe with 6-OHDA solution instead of LV stock

   - In step 15: we inject 6-OHDA at the same locations as vector (*see* **Note** 10)

   - In step 20: discard remaining 6-OHDA

3. Timing of 6-OHDA lesioning (*see* **Note** 11)

3.4. *Post-mortem* brain processing

1. Sacrifice rats by CO_2 exposure and decapitate
2. Open the skull with medium scissors or forceps, remove the whole brain and transfer into 50 ml falcon tubes filled with ice-cold 4% PFA (1 brain/tube, *see* **Note** 12)
3. Fix brains for 3-5 days at 4°C
4. Rinse brains with ice-cold 1x PBS
5. Slice brains on a vibrating microtome at 50 μm thickness
6. Collect brain sections using a paint brush and keep in ice-cold 1x PBS during sectioning
7. Wash in ice-cold 1x PBS (2 x 2 min) with gentle agitation
8. Block in 1% BSA blocking buffer for 1 h, RT
9. Incubate with primary antibody overnight, 4°C (*see* **Note** 13)
10. Wash in 1x PBS (3 x 5 min) with gentle agitation
11. Incubate with compatible secondary antibody for 1 h, RT (*see* **Note** 13). Protect samples from light after this step
12. Wash in 1x PBS (3 x 5 min) with gentle agitation
13. Incubate with DAPI (1 μg/ml) for 15 min, RT
14. Wash in 1x PBS (3 x 5 min) with gentle agitation
15. Use paint brush to flatten brain sections onto SuperFrost slides (~3 sections/slide)
16. Mount with mounting solution (50-100 μl/slide) and cover with coverslips
17. Air dry at RT and store at 4°C

4. Notes

1. Mowiol may not be dissolved completely but the pellet must be colourless. After centrifugation, collect and aliquot the supernatant and store at -20°C until use. Do not disturb the pellet.
2. As growth rate of 293T cells is quite variable, adjust cells seeded accordingly.
3. Plasmid stocks and reagents are endotoxin-free and tissue culture grade. LV plasmids are self-inactivating and contain a central polypurine tract/central termination sequence and Woodchuck hepatitis virus post-transcriptional regulatory element.
4. Replace culture medium again with 18 ml/plate of fresh medium for second harvest (after additional 24 h), if required. The first harvest usually provides the highest vector titres but the second harvest can have comparable yield.
5. We favor titration of the late reverse transcript by qPCR ***(20)*** in transduced cells harvested 24 h post-transduction, normalizing with the amount of endogenous β-actin gene, as described ***(9)*** and discussed ***(8)***. It is important to harvest transduced cells at 24 hours when titrating IDLVs (and matched IPLV stocks) by qPCR, to minimize loss of episomes due to cell proliferation-mediated dilution.
6. We obtained successful LV transduction with widespread *eGFP* expression within the striatum following 2 injections at: (1) AP: +1.8 mm, ML: -2.5 mm relative to bregma and DV: -5.0 mm relative to dura; (2) AP: 0.0 mm, ML: -3.5 mm relative to bregma and DV: -5.0 mm relative to dura. Vectors were injected at 5 μl/site, 10^9 viral qPCR transducing units/ml (*see* **Note 5**).
7. Two ear bars should be inserted at equivalent depth, usually around 7. If the bars are set at the right position, when you release incisors from the bar and use your index

finger to gently press the head down or lift it up, the head will move rigidly and not drift downwards.

8. Be careful not to confuse bregma with lambda (just below bregma).
9. Pargyline and desipramine are used to increase the bioavailability and specificity of 6-OHDA for dopaminergic neurons.
10. We used 6-OHDA at 2.5 μg/μl and injected 2 μl/site at two sites into the striatum and observed 50% death of dopaminergic neurons in the ipsilateral substantia nigra.
11. 6-OHDA lesioning can be administered before or after LV injection depending on the purpose of the study.
12. The volume of 4% PFA should be 10-20 times that of the brain for complete fixation. We used 20 ml/brain.
13. Dilute antibodies in blocking buffer at concentrations according to manufacturers' recommendation or previous optimization.

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