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Improvement of Neurological Dysfunctions in Aphakia Mice, a Model of Parkinson's Disease, after Transplantation of ES Cell-Derived Dopaminergic Neuronal Precursors

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Abstract

Parkinson's disease (PD) is characterized by selective death of the substantia nigra dopaminergic neurons, and previously we have shown that *aphakia* mice, which harbor spontaneous Pitx3 gene mutation, show specific degeneration of the substantia nigra dopaminergic neurons accompanied by behavioral deficits that is reversed by L-DOPA treatment or transplantation of dopaminergic neural precursors. Here, we describe transplantation of dopaminergic neural precursors to a mouse model of PD, an *aphakia* mouse, followed by behavioral analyses of transplanted mice.

Keywords

Embryonic stem cells; Dopamine; Neuronal precursors; *Aphakia* mice; Parkinson's disease; Transplantation

1 Introduction

There are several animal models of Parkinson's disease (PD), and among these, an *aphakia* mouse is a genetic model [1, 2] that can provide a homogeneous model system compared to the pharmacologically induced models. Unlike many other genetic models of PD [3, 4], *aphakia* mice show selective degeneration of the substantia nigra dopaminergic neurons [1, 5–7] as in the case of human diseases, accompanied by behavioral deficits, which can be reversed by pharmacological treatment [2] or cell transplantation [8, 9]. Here, we describe the generation of dopaminergic neuronal precursors from mouse embryonic stem cells for transplantation, stereotaxic surgery of *aphakia* mice to deliver dopaminergic neuronal precursors into the striatum, and the behavioral analyses of the transplanted *aphakia* mice using various assays such as challenging beam test and pole test.

2 Materials

2.1 In Vitro Differentiation of Embryonic Stem (ES) Cells

1. Gelatin-coated dish: Incubate a 10 cm tissue culture plate with 1 mL of 0.1 % Gelatin solution (STEMCELL Technologies) at room temperature for 30 min (*see* Note 1).
2. ES cell medium: Dulbecco's modified Eagle medium (DMEM) supplemented with 2 mM glutamine (Invitrogen), 0.001 % β -mercaptoethanol, 1 $\times$ nonessential amino acids (Invitrogen), 10 % donor horse serum (Sigma), and 2,000 U/mL human recombinant leukemia inhibitory factor (LIF; R&D Systems).
3. Trypsin–EDTA: diluted to 1 $\times$ with Ca_2^+ -free, Mg_2^+ -free phosphate-buffered saline (PBS).
4. Embryoid bodies (EB) medium: DMEM supplemented with 2 mM glutamine, 0.001 % β -mercaptoethanol, 1 $\times$ nonessential amino acids, 10 % fetal bovine serum (FBS).
5. Non-adherent bacterial dishes (Fisher Scientific).
6. Insulin, transferrin, selenium, and fibronectin (ITSFn) medium: DMEM/F12 (Invitrogen) supplemented with 50 $\mu\text{g/mL}$ transferrin (Sigma), 5 $\mu\text{g/mL}$ insulin (Sigma), 30 nM sodium selenite (Sigma), and 500 ng/mL fibronectin (Sigma).
7. Poly-L-ornithine and fibronectin-coated coverslips: Incubate sterile glass coverslips in Poly-L-ornithine (PLO; 15 $\mu\text{g/mL}$; Sigma) and fibronectin (FN; 1 $\mu\text{g/mL}$; Sigma) at room temperature for 30 min (*see* Note 2).
8. Neuronal progenitor (NP) medium: DMEM/F12 supplemented with N2 supplement (Invitrogen), 1 $\mu\text{g/mL}$ laminin (Sigma), and 10 ng/mL basic fibroblast growth factor (bFGF; R&D Systems).
9. Neuronal differentiated (ND) cell medium: DMEM/F12 supplemented with N2 supplement and 1 $\mu\text{g/mL}$ laminin.
10. FACS medium: phenol-free, Ca_2^+ -free, Mg_2^+ -free Hank's balanced salt solution (HBSS) supplemented with 1 $\times$ penicillin/streptomycin, 20 mM D-glucose, and 2 % FBS.
11. Anti-Corin antibody [¹⁰]: a kind gift from Bruce A. Morgan, Harvard Medical School/Massachusetts General Hospital, Boston.
12. Alexa Fluor 647-conjugated anti-rabbit antibodies (Invitrogen).
13. FACS Aria cell sorter with FACSDiva software (BD Biosciences).

¹Gelatin-coated plates can be made in bulk and stored at 4 °C until needed.

²Poly-L-ornithine and Fibronectin can be mixed together for 30 min coating, or can be incubated for coating serially. Extra coverslips coated with Poly-L-ornithine and Fibronectin can be stored at 4 °C until needed.

2.2 Stereotaxic Surgery

1. Transplantation medium: phenol-free, Ca_2^+ -free, Mg_2^+ -free HBSS supplemented with 10 ng/mL brain-derived neurotrophic factor (BDNF) and 10 ng/mL glial cell-derived neurotrophic factor (GDNF).
2. Pre-anesthesia: acepromazine (3.3 mg/kg, PromAce) and atropine sulfate (0.2 mg/kg, Phoenix Pharmaceuticals).
3. Anesthesia injection: Mix 0.6 mL ketamine (100 mg/mL; Sigma) and 0.15 mL xylazine (20 mg/mL; Phoenix Pharmaceuticals) with 9.25 mL saline, and use 0.1 mL per 10 g body weight.
4. Kopf stereotaxic frame (Kopf Instruments) with mouse adaptor.
5. Betadine.
6. Disposable scalpel.
7. Micro drill sets (Fine Scientific Tools).
8. 5 μL Hamilton syringe with 2' 28-gauge needle with beveled end.

2.3 Behavioral Analyses

1. Challenging beam test: 1 m beam that starts at a width of 3.5 cm and gradually narrows to 0.5 cm in 1 cm decrements with wire-grid surface (1 cm square).
2. Pole test: 50 cm length round wooden pole vertically fixed on a styrofoam platform.

3 Methods

3.1 In Vitro Differentiation of ES Cells (ESCs)

1. Culture undifferentiated Otx2GFP KI ESCs [¹¹] on gelatin-coated dishes in ES cell medium.
2. Differentiate ESCs into embryoid bodies (EBs) on non-adherent bacterial dishes for 4 days in EB medium.
3. Transfer EBs onto adhesive tissue culture plate. Twenty-four hours after transfer, initiate selection of neuronal progenitor (NP) cells in serum-free ITSFn medium (see Note 3).
4. After 10 days of selection, trypsinize cells for FACS isolation of dopaminergic neuronal precursors.
5. Trypsinize differentiated cells at the end of stage 3, gently triturate and label by incubating with an anti-Corin antibody in FACS medium for 30 min at 4 °C, followed by incubation for 15 min with Alexa Fluor 647-conjugated anti-rabbit

³To increase the proportion of midbrain dopaminergic neuronal precursors, recombinant sonic hedgehog (SHH; 100 ng/mL; R&D systems) or chemical activators of SHH signaling such as Purmorphamine (2 μM ; Cayman Chemicals) can be added at the selection stage in ITSFn media [8].

antibodies in FACS medium. Perform all washing steps in FACS medium. Filter stained cells through cell strainer caps (35 mm mesh) to obtain single-cell suspensions (10^7 cells/mL for sorting).

6. Sort stained cells on a fluorescence-activated cell sorter, FACSAria, using FACSDiva software (*see* Note 4). Forward- and side-scatter gating identify the population of interest, excluding debris and dead cells. Corin positivity will be determined according to negative controls consisting of absence of primary antibody and absence of primary and secondary antibodies, whereas GFP positivity will be determined by comparing J1 ESC-derived cells lacking GFP expression. Before sorting, the nozzle, sheath, and sample lines should be sterilized with 70 % ethanol or 2 % hydrogen peroxide for 15 min, followed by washes with sterile water to remove residual decontaminant.
7. Plate sorted OtxGFP⁺ Corin⁺ NP cells on PLO/FN-coated tissue culture plate or coverslips in NP medium for transplantation or in vitro analysis, respectively. After 4 days of expansion in NP medium, initiate differentiation by changing medium to ND medium for in vitro analysis.

3.2 Stereotaxic Surgery

1. Trypsinize FACS-purified Otx2GFP⁺ Corin⁺ cells, expanded for 4 days in NP medium, and suspended to a final concentration of 50,000 cells/ μ L in transplantation medium (*see* Note 5).
2. Anesthetize the mice by administering (i.p.) a mixture of acepromazine and atropine sulfate, as pre-anesthetics followed by intraperitoneal administration of ketamine and xylazine.
3. Test depth of anesthesia by foot pinch. If needed (i.e., animal responding to foot pinch), inject half a dose of anesthetics, followed by retesting the animals by foot pinch after 10 min.
4. Load 2 μ L of cell solution into a Hamilton syringe and fasten to the stereotaxic frame (*see* Note 6).
5. Remove the hair from the surgery area of anesthetized mice, and then place them on the stereotaxic equipment with the mouse adaptor. Secure the skull with ear bars, and then apply betadine to the surgical area and make an incision using a disposable scalpel (*see* Note 7) exposing the skull, which will be wiped with betadine.

⁴In general, we start with approximately 5×10^6 ES cells for in vitro differentiation, resulting in approximately $2-4 \times 10^8$ mixed NP cells at the end of stage 3. Thus, allowing for a certain degree of cell loss during staining and FACS, we usually recover $2-4 \times 10^6$ viable double-positive cells.

⁵Adding Boc-Asp(OMe)-fluoromethyl ketone (BAF) to the transplantation medium increases post-transplantation cell viability mildly but significantly [12].

⁶Prior to loading the Hamilton syringe with cell solution, make sure to flush the syringe with 70 % ethanol, followed by rinsing with sterile water and then with PBS and make sure that the needle is not clogged. When taking up the cell solution, avoid air bubbles (keep the entire beveled end of the needle in the liquid). Even though 1 μ L will be used for injection, taking up 2 μ L of cell solution makes it easier to dispense exactly 1 μ L of cells.

⁷If the animal reacts to the scalpel blade, pause the surgery and inject another half dose of anesthesia, wait 10 min and retest by foot pinch before resuming surgery.

6. After identification of the stereotaxic coordinate of bregma, calculate the injection site coordinates and mark the injection site (*see Note 8*).
7. Using a micro drill make a burr hole at the injection site.
8. Lower the end of the needle to the surface of the dura, and measure ventral coordinate of dura to calculate the coordinate of injection site based on that. Lower the needle to the injection site and slowly inject 1 μ L of cell solution at a rate of 0.5 μ L/min (*see Note 9*).
9. Upon completion of the injection, leave the needle at the injection site for 2 more min to allow time for the cell solution to diffuse. After 2 min, pull the needle up slowly.
10. Remove the mice from the stereotaxic frame and transfer them to a warm heat pad for suture and recovery. After applying betadine solution to the suture site, also apply antibiotic ointment to the area. Also, if needed, ear punch the mice for identification.
11. Upon awakening, transfer the mice to their original cage (*see Note 10*).

3.3 Behavioral Analyses¹

1. Perform nigrostriatal pathway-sensitive motor behavioral tests such as challenging beam and pole tests (*see Note 11*) 4 weeks and 6 weeks post transplantation, using mock-transplanted *aphakia* mice and blind retinal degeneration 1 (*rd1*) mutation mice as controls (*see Note 12*).
2. For the pole test, place the animals head upward on top of a vertical wooden pole (50 cm in length and 1 cm in diameter) with the base of the pole being placed in the home cage. Once on the pole, the animals orient themselves downward and descend the length of the pole back into their home cage. Train all the animals for 2 days consisting of three trials at each session. On the test day, the animals receive three trials, and the times to orient downward and total travel time are measured (Fig. 1a, b) (Reproduced from ref. 8 with permission from PNAS).
3. For the challenging beam traversal test, measure motor performance with the beam test as described previously [²]. The beam (length, 1 m) starts at a width of 3.5 cm and gradually narrows to 0.5 cm in 1 cm decrements with wire-grid surface (1 cm wire). Train the animals for 2 days to traverse the length of the beam, starting at the widest section and ending at the narrowest section (*see Note 13*).

⁸We used the following injection site coordinates from bregma: AP + 0.05, L $\pm$ 0.18, V -0.30, IB 9.

⁹It is important to inject the cell solution slowly to minimize tissue damage caused by the pressure of the sudden injection.

¹⁰If male mice are used, especially make sure to return the mice to their original cage and littermate to avoid fighting.

¹¹As an additional test for the nigrostriatal pathway-sensitive motor behavioral test, a cylinder test can be performed. Spontaneous movement is measured using a small transparent cylinder (height, 15.5 cm; diameter, 12.7 cm). Mice are placed in the cylinder for 3 min and the number of rears is measured. A rear is defined as an animal's vertical movement with both forelimbs and immediately touching the wall of the cylinder after removing both limbs from the ground. (Reproduced from ref. 8 with permission from Cell Transplant.)

¹²Since *aphakia* mice are born blind, we used blind *rd1* mice as control for these behavioral tests, in addition to sham treated *aphakia* mice.

¹³Use gentle prodding if necessary during training, until the mice cross the bar readily. Sometimes putting bedding from the home cage at the end point helps.

4. While traversing the grid-surfaced beam for a total of three trials, video record the animals and measure times for each animal to traverse as shown in Fig. 1c (Reproduced from ref. 8 with permission from PNAS) (*see* Note 14).

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¹⁴As independent variables, the number of slips and the number of steps taken to traverse could be measured after slow speed playback of the videotape. To measure motor learning, the mean of the slope difference from training day 1 to the testing day (performance on the testing day minus that on the first training day) can be analyzed.

Fig. 1. Otx2GFP + Corin + midbrain dopaminergic neuronal precursors, when transplanted into *aphakia* mice, can ameliorate the behavioral deficit. *Rd1* mice were used as a blind mice control. **(a, b)** Pole test. **(c)** Challenging beam test. (Mean $\pm$ S.E.M.; $n = 10$)