

Transplantation of Embryonic Ventral Mesencephalic Tissue in 6-OHDA Induced Parkinsonism Rat Brain for Cell Based Therapy: A Perspective of Methods

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To cite this article:

Biswarup Ghosh, Angelo Lepore, George Smith. Transplantation of Embryonic Ventral Mesencephalic Tissue in 6-OHDA Induced Parkinsonism Rat Brain for Cell Based Therapy: A Perspective of Methods. *International Journal of Genetics and Genomics*. Vol. 4, No. 6, 2016, pp. 79-84. doi: 10.11648/j.ijgg.20160406.16

Received: July 18, 2016; Accepted: July 29, 2016; Published: March 22, 2017

Abstract: Parkinson's disease (PD) is characterized as a disease of the basal ganglia, with a progressive degeneration of dopaminergic neurons located in the substantia nigra (SN) and projecting to the striatum with subsequently loss of the nigrostriatal circuit. The potential for therapeutic use of cell transplantation for cell replacement has received a great deal of interest. Transplantation with embryonic ventral mesencephalon (VM) is a therapeutic approach for sporadic form of PD. We established unilaterally 6-OHDA lesioned rat model of Parkinson's disease. Motor behavioral impairment was found compared with normal rat. Embryonic VM tissue was isolated from E 14 (embryonic 14 days) rat brain. We characterized the VM tissue and dissociated cultured dopaminergic cells with tyrosine hydroxylase (TH) immune staining before transplantation in lesioned brain. We observed that the axons of dopaminergic neurons from transplanted VM graft circles round at the site of transplantation in normal adult rat brain. In this paper, we discuss the detailed methodologies which are very useful in preclinical research of cell based therapies for Parkinson's disease.

Keywords: Transplantation, 6-OHDA, Parkinson's Disease

1. Introduction

Loss of nigrostriatal pathway is the predominant cause of Parkinson's disease and the selective degeneration of nigrostriatal dopaminergic neurons made neuronal transplantation a potential therapy for Parkinson's disease. Indeed, neuronal transplantation has been widely used in animal models in laboratories and clinical trials. This loss of dopamine within the striatum results in motor dysfunction, including resting tremor, muscular rigidity, bradykinesia and postural instability. To date, no therapies have been developed to reverse or slow down the progression of this illness. Typically, transplantation mechanisms for repair in PD utilized human foetal graft techniques, in which embryonic dopaminergic cells are harvested from the developing midbrain. The clinical outcomes of these studies have been mixed but show good transplant survival in

striatum and integration of grafted dopaminergic neurons [1-4]. Some trials evaluating human foetal grafting raised significant concerns about this technique with a number of patients developing 'off'-medication dyskinesias [5; 6].

The neurotoxin 6-hydroxydopamine (6-OHDA) has played a fundamental role in preclinical research on sporadic form of Parkinson's disease (PD). 6-OHDA is a structural analogue of catecholamines, dopamine and noradrenaline, and exerts its toxic effects on catecholaminergic neurons. Neurotoxic effects of 6-OHDA occur through accumulation of the free radicals in catecholaminergic neurons, followed by alteration of cellular homeostasis and neuronal damage. The uptake and storage of 6-OHDA is mediated by the dopamine or noradrenaline membrane transporters (DAT and NAT respectively) due to its structural similarity with endogenous catecholamines. Oxidative stress is the key factor for 6-OHDA-induced neuronal damage [7; 8]. Oxidation of 6-

OHDA by monoamine oxidase (MAO-A) generates hydrogen peroxide (H_2O_2) which triggers the production of oxygen radicals [9]. Moreover, 6-OHDA undergoes robust auto-oxidation generating cytotoxic H_2O_2 , reactive oxygen species (ROS) and catecholamine quinones which attack endocellular nucleophilic groups [10; 11]. 6-OHDA increases ROS and other reactive species levels resulting in a rapid depletion of endocellular antioxidant enzymes, leading to an amplified neurotoxicity causing abnormalities in cell structure and metabolism and eventually resulting in neuronal damage [8].

Unilateral 6-OHDA model is the traditional model for testing Parkinson's therapies, especially those intended to increase dopamine levels in the striatum. The principal advantage of this model is that it is very sensitive to dopamine agonists. The toxin 6-OHDA is injected unilaterally, while the opposite hemisphere serves as an intra-animal control. This injection produces DA neuron loss on the 6-OHDA-injected side while sparing the contralateral DA neurons.

In this communication we discussed the detailed methodologies which includes 1) generation of 6-OHDA induced rat model of Parkinson's disease, 2) motor behavioral assessment, 3) dissection of embryonic E14 VM tissue and dopaminergic cell culture, 4) stereotactic transplantation of tissue graft in brain and 5) immunostaining for dopaminergic marker TH. These are very useful for new researchers and for preclinical research of cell based therapies of sporadic form of Parkinson's disease.

2. Methods

2.1. Animals

Adult female Sprague-Dawley rats (225-250g) were obtained from Harlan Laboratories (Indianapolis, IN). Rats were housed two animals in a cage with sterile food and water in Temple University's Division of Laboratory Animal Resources facility. All procedures were conducted in strict compliance with approved of each institutional protocol and in accordance with the provisions for animal care and use described in the Guide for the Care and Use of Laboratory Animals.

2.2. 6-OHDA Lesioning

Adult female Sprague-Dawley rats were given unilateral 6-OHDA lesions of the nigrostriatal pathway as follows. Rats were anesthetized with a mixture of ketamine (67 mg/kg, i.p.) and xylazine (6.7 mg/kg, i.p.) and positioned in a stereotaxic frame. The skull was exposed and two holes were drilled with the coordinates (AP-4.4, ML 1.2 relative to bregma and AP -5.3, ML 2.0). Two injections of $3.0\mu\text{g}/\mu\text{l}$ 6-OHDA in 0.9% saline with 0.2% ascorbic acid were made at a rate of $1.0\mu\text{l}/\text{min}$ for 2 min, in the vicinity of the medial forebrain bundle (AP-4.4, ML 1.2 relative to bregma, 8.4mm deep from skull level) and the rostral substantia nigra pars compacta (AP -5.3, ML 2.0, 8.4mm deep). This procedure has been shown to produce complete lesions, with near

complete dopaminergic denervation of the ipsilateral striatum. This denervation was confirmed 4-5 weeks after lesioning by amphetamine-induced rotation testing (>600 ipsilateral turns in 90 minutes).

2.3. Amphetamine-Induced Rotation

Four and five weeks after lesioning, rats were injected with 5mg/kg D-amphetamine in 0.9% saline (i.p.). Immediately after injection, each animal was placed in a harness and enclosed in a clear plastic cylinder as part of the Rotamax-8 apparatus (Columbus Instruments, Columbus, OH). The number of partial and full clockwise and counterclockwise rotations made by each rat in a 90-minute test period was automatically recorded on an attached computer with Rotacount version 2.0 software.

2.4. Cylinder Tests

Animals determined to have complete lesions were further tested for limbuse asymmetry during spontaneous motor behavior using the cylinder test described by Schallert and Tillerson (1999) [12]. Rats were placed individually into a clear plastic cylinder and videotaped for 3-5 minutes – as long as it took each one to touch the cylinder wall with a forelimb at least 20 times during spontaneous exploration of the environment. Videotapes were analyzed by a person blinded to experimental design, and the number of wall touches made with the left, right or both forepaws were tallied. A limb use asymmetry score was determined by dividing the number of touches with the left paw by the total number of wall touches (left, right, or both paws together). A score of one would indicate complete asymmetry with use of left forepaw only.

2.5. Ventral Mesencephalic Tissue Dissection and Transplantation

E14 embryos were removed from a pregnant Sprague-Dawley rat and the VM brain region (approximately $1\text{mm} \times 1.5\text{mm} \times 1\text{mm}$) was dissected from each fetus and kept in ice cold calcium- and magnesium-free Hibernate buffer (BrainBites, IL) until transplantation. Rats were anesthetized with a mixture of ketamine (67 mg/kg, i.p.) and xylazine (6.7 mg/kg, i.p.) and placed in a stereotaxic apparatus, a hole was drilled at -5.2mm AP and 2.4mm ML on the left side relative to bregma. $0.2\mu\text{l}$ Chondroitinase ABC (ChABC) was injected at the transplantation site right before VM tissue transplantation to reduce the possible over-expression of CSPG induced by damage from the transplantation needle as we observed in our corpus callosum pathway model [13]. The E14VM tissue from a single fetus was drawn into the blunt end of a 22-gauge spinal needle. The needle was lowered to -7.8 mm from the dural surface, then pulled up to -7.5 mm before ejecting the tissue. Each tissue chunk was ejected slowly by depressing the needle's plunger ~1mm every 20 seconds. The needle was kept in place for 5 minutes after ejection, raised 0.4mm and kept there for another 5 minutes before slowly raising it all the way out of the brain to

be sure the transplant remained in place.

2.6. Dopaminergic Cell Culture

Embryos at day 14 (E14) were removed from a pregnant Sprague-Dawley rat and the ventral mesencephalic (VM) brain region was dissected out of each fetus and kept in ice cold, sterile, calcium- and magnesium-free buffer. The tissue chunks were rinsed in fresh buffer several times, trypsinized (0.125% trypsin) for 10 minutes at 37°C, and then rinsed again. Cells were then dissociated by trituration in 0.04% DNase, layered over sterile FBS and pelleted out by centrifugation. The resulting cell pellet was resuspended in Dulbecco's modified Eagle Medium (DMEM)/Ham's F12 (DMEM/F12, 1:1) with 5% fetal bovine serum (FBS) and 10U/ml penicillin, and cell density and viability was determined with trypan blue and a hemocytometer. Twenty microliters of the E14 VM cell suspension (~100,000 cells) was then added to each poly-D-lysine coated coverslip placed in six o plate well.

2.7. Tissue Preparation and Immunohistochemistry

Animals were perfused with ice-cold saline followed by 4% paraformaldehyde (PFA) in phosphate buffer. Brains were carefully removed and post-fixed overnight in 4% PFA at 4°C then placed in a 30% sucrose solution at 4°C until brains sank to the bottom of the vials (2-3 days). Brains were then embedded in Tissue-Tek OTC compound, frozen on dry ice, and sliced at 30µm horizontally and or coronal with a cryostat. Brains were divided into six sets of serial sections for subsequent immunostaining.

Immunohistochemistry procedures were carried out at room temperature. The free floating sections were quenched for 10 minutes in 3% H₂O₂ and washed with PBS. The cultured dopaminergic cells were prefixed with 4% PFA for 10 mins. Cells/Sections were then blocked with 10% normal goat serum (NGS) in PBS for 1 hour and incubated overnight in a mouse primary antibody against tyrosine hydroxylase (TH; 1:4000 dilution; Millipore/Chemicon), followed by a 1 hour incubation in biotinylated secondary antibody (biotin-SP-conjugated affinipure goat anti mouse IgG 1:600, Jackson). Cells/Sections were then incubated for 1 hour in a streptavidin-horseradish peroxidase complex (ABC Elite kit, Vectastin, Vector laboratories) in Tris-buffered saline-Triton (TBST, 0.04%). After that, the cells/sections were exposed to di-amino-benzidine (Nowak, Szczerbak et al.) (0.5mg/ml, Sigma plus 3% H₂O₂), followed by washing in Tris-buffer. The free floating sections were mounted on gelatin-coated slides and dehydrated in alcohol and CitriSol before being coverslipped with Permount. The cells were dehydrated in alcohol and coverslipped with Permount.

2.8. Statistical Analyses

Data are reported as mean ± SE. Comparisons between two groups were performed with unpaired Student's t-tests. One-way ANOVA was performed whenever appropriate. A p value of less than 0.05 was considered statistically

significant.

3. Results

3.1. Characterization of Unilateral Lesion

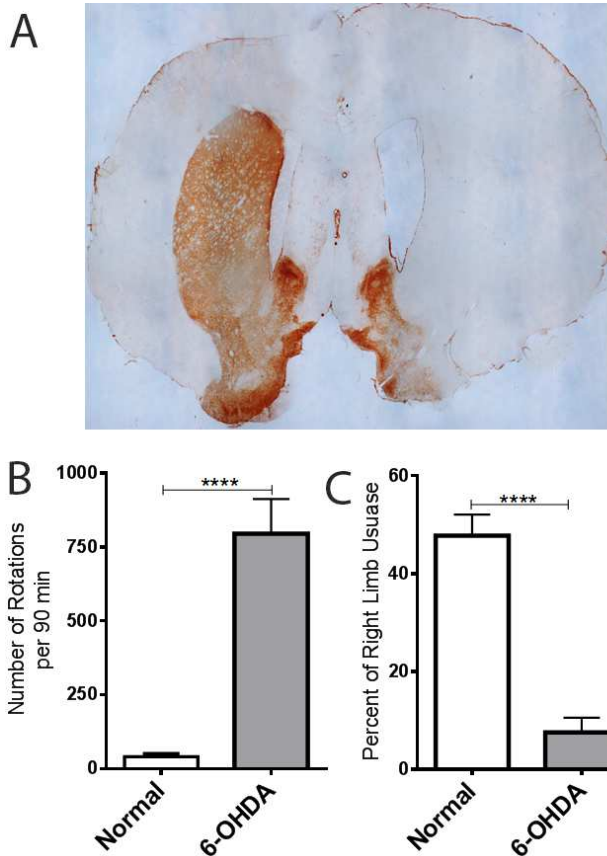


Figure 1. 6-hydroxydopamine (6-OHDA) induced unilateral lesions in rat brain A) Coronal section of a similarly unilateral lesioned rat brain immunostained with TH (10X magnification). B) Amphetamine-induced rotational assessment of rats one month after 6-OHDA treatment showed a significant (unpaired T-test, $p < 0.0001$) increase in rotations compare to untreated rats. C) Forelimb use asymmetry test 1 month after lesioning shows a significant (unpaired T-test, $p < 0.0001$) in right limb (contralateral to 6-OHDA injection) use when compared to untreated rats.

To establish the 6-OHDA induced hemiparkinsonium rat model, we used 12 µg doses of 6OHDA for complete ablation as determined by histopathology and motor movement. We also choose to inject 6OHDA into both the medial forebrain bundle and substantia nigra to ensure complete destruction of A9 dopaminergic neurons. Unilateral Injection induced a complete loss of A9 and partial loss of A10 dopaminergic neurons within midbrain and an almost complete denervation of TH+ axons from the striatum (Fig. 1 A).

3.2. Motor Behavioral Impairment

Motor impairment was examined using both D-amphetamine induced rotations and spontaneous motor behavior for paw placement onto the walls of a glass cylinder. Complete lesions resulted in increased d-

amphetamine induce rotations (>700 rotations/90 mins; Fig. 1B) when compared to control non-lesioned animals. However, complete lesions showed a statistically significant reduction in limb usage and placement of the contralateral forepaw (Fig. 1C).

3.3. Characterization of VM Tissue

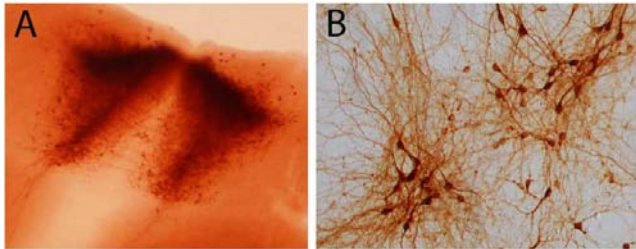


Figure 2. Characterization of embryonic ventral mesencephalic (VM) tissue. A) TH staining of VM tissue isolated from E14 pups. B) TH staining of dopaminergic neurons in culture obtained from VM tissue.

Ventral mesencephalic tissue immunostained for TH clearly shows the position of the dopaminergic embryonic precursor cells. E14 VM tissue contains precursor cells of dopaminergic neurons (Fig. 2A). Dopaminergic neurons were labeled when dissociated cells from the VM tissue were cultured in vitro (Fig. 2B).

3.4. Transplant Survival

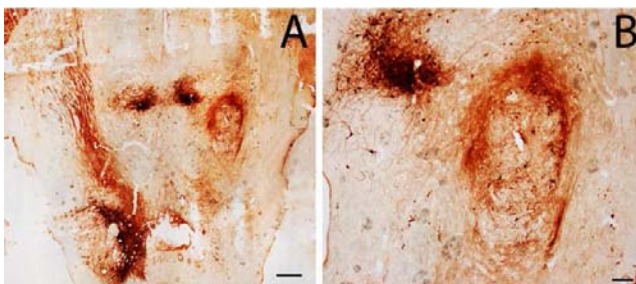


Figure 3. VM tissue graft transplantation in 6-OHDA induced parkinsonism rat brain. A) Horizontal section stained with TH staining showed transplanted dopaminergic neurons. B) High magnification image of the transplanted dopaminergic neurons. Scale bar: 200 μ m (A), 100 μ m (B).

Animals were euthanized and brain tissue harvested 8 weeks after transplantation and after all behavioral analyses were completed. With immunostaining for tyrosine hydroxylase (TH), transplants were labeled (Fig 3A). Dopaminergic neurons differentiated from embryonic VM tissue at the site of transplantation within the lesioned brain. It was evident that axons failed to grow out of transplants and circles round the site of transplantation (Fig. 3B).

4. Discussions

Experimental transplantation of exogenous neurons into adult mammalian brains was reported in the late 1800's, but was not pursued as a potential therapy for CNS disorders until the 1970's [14]. Transplanted cells may confer therapeutic benefits in two different ways: 1) by forming

functional, reciprocal connections within the host brain or spinal cord, essentially replacing lost neural circuits, or 2) by producing neurotransmitters or neurotrophic/neuroprotective factors to enhance survival, regeneration and function of spared host neurons [14; 15]. Cells used for transplantation are always immature: derived either from embryonic dissections or in vitro expansions of neural stem/progenitor cells. As discussed in the previous sections, their immature state means these cells are more likely to survive, grow, respond to environmental cues and successfully integrate into the host CNS. Investigations into neural transplantation therapies are ongoing for many neurological disorders, including spinal cord injury, stroke, amyotrophic lateral sclerosis, Huntington's disease, and Parkinson's disease.

Research into cell transplantation for Parkinson's disease (PD) began in the 1970s, when it was first determined (using intraocular grafts and then brain grafts) that embryonic neural tissue was best suited for surviving transplant procedures, extending neurites into host tissues, and forming synaptic connections [16; 17]. One problem that was encountered at the time was insufficient graft survival, mainly due to a lack of blood supply to the cells within a piece of transplanted tissue. The development of a procedure to dissociate appropriate neurons into a suspension and deliver them to the host brain by stereotaxic injection helped to alleviate this problem [18]. Our studies had shown that the cells differentiated from VM transplant in brain usually were dopaminergic neurons (Fig. 2 & 3). Most successful transplant studies, including human clinical trials, involve grafting of embryonic VM cells directly into the striatum of the host. This ectopic graft placement avoids the problem of how to direct axonal growth long distances from the original location (the substantia nigra, SN) to the appropriate target (striatum). It has been shown in many laboratories that VM transplants into the striatum of Parkinsonian animals can survive long-term, extend neurites into surrounding host tissue, make synaptic connections, respond to afferent stimulation, express enzymes for dopamine synthesis, release dopamine, and reverse some behavioral deficits [19; 20]. The survival of transplanted VM neurons is variable, however, and has been enhanced with various techniques, including repeated injections of GDNF adjacent to grafts [21], co-transplant of neurospheres modified to produce GDNF adjacent to grafts [22], and ex vivo transduction of neurons with cDNA encoding human vascular endothelial growth factor prior to transplant [23]. Graft survival and striatal reinnervation were also improved by using a multiple-site microinjection technique to distribute the cells over a wider area [24].

To explore the role of dopaminergic input to the SN itself, intranigral transplants of fetal VM cells have been carried out in 6-OHDA-lesioned rats [25]. While early attempts at this procedure failed due to the small transplant site and traumatic transplantation procedures, Nikkiah and colleagues used a microtransplantation approach that resulted in extensive reinnervation of the SNr and some functional recovery [26]. This study showed that intranigral grafts decreased rotational

asymmetry caused by injection of dopamine agonists, but not that caused by amphetamine. Other studies of intranigral VM transplantation have confirmed the attenuation of dopamine agonist-induced turning, and shown that some more complex behaviors may be partially restored [27; 28]. Intranigral transplants performed in MPTP-treated monkeys resulted in a “modest but detectable benefit” [29]. To determine whether reinnervation of both the SN and the striatum could result in additive functional benefits, some groups have performed simultaneous intrastriatal and intranigral grafts. This method seems to provide more behavioral benefits than striatal grafting alone, and one pilot clinical trial has already determined the safety and efficacy of double grafts in human PD patients [27; 30-32].

To truly restore the circuitry that is lost in PD, neurons that are transplanted into the substantia nigra should extend axons to the striatum. The distance between the SN and the striatum, coupled with the inhibitory environment of adult CNS, makes it difficult to effect such axon growth [33]. Successful reconstruction of the nigrostriatal pathway was accomplished in neonatal (postnatal day 3) rat brains previously lesioned with 6-OHDA, using rat embryonic VM, but this has little clinical relevance, since PD only affects adults [34]. In the adult rat, only xenografts of human or porcine tissue into the SN have resulted in some unaided long distance axonal growth within the medial forebrain bundle and the internal capsule, specifically toward and then into the striatum [35-37].

The use of human fetal tissue as a source for transplantation has always posed a number of ethical and practical concerns and it is possible that these could be avoided by the use of human embryonic stem (ES) cells or induced pluripotent stem (iPS) cells. ES cells could potentially provide an unlimited source of human midbrain dopaminergic neurons with appropriate neural precursor cell selection and culture techniques. For evaluation of the functional effect of cell based transplantation, researchers may need some refinement of the methodologies and the methods described in this paper will help designing proper model of study and evaluation of motor behavioral study.

In future our lab will focus to reconstruct the naïve nigrostriatal circuit by nigral transplantation of embryonic VM tissue. Axons will be targeted into striatum within the medial forebrain bundle and the internal capsule by creating viral mediated highway of neurotrophic factors. Unilateral complete lesion model preparation is very important in this kind of regeneration studies within brain. Incomplete lesion containing spared axons will interfere the efficacy of functional outcomes after VM tissue transplant.

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