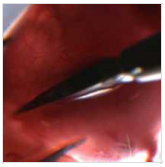

Jul 21, 2021

Transplantation of Fetal Midbrain Dopamine Progenitors into a Rodent Model of Parkinson's Disease

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ABSTRACT

Cell therapy is a promising experimental treatment for Parkinson's disease (PD). It is based on the idea that new dopamine neurons transplanted directly into the forebrain of the patient can structurally and functionally compensate for those lost to the disease in order to restore motor function. While there is a highly active field of research focused on the development of stem cell-based procedures, fetal tissue remains the "gold standard" as a safe and reliable source of dopamine neuron progenitors capable of structural and functional integration with existing motor circuitry following transplantation. This chapter describes the basic procedures for preparation of dopamine progenitor rich cell suspensions of ventral mesencephalon as well as implantation into the unilateral 6-hydroxydopamine model of PD and assessment of functional impact according to drug-induced rotational behavior. The description assumes a basic knowledge of animal handling and stereotaxic surgical procedures in rodents.

ATTACHMENTS

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1 Introduction

The inability of the brain to repair itself makes *neural transplantation* an attractive approach for the treatment of neurological conditions. Although the concept has been explored since as early as the late 1800s [1], it gained momentum in the 1970s following studies showing that intra-cerebral grafts of fetal ventral mesencephalon (VM) could restore motor function in a rodent model of Parkinson's disease [2, 3]. These early experiments relied on the use of small VM tissue pieces that were placed in the adult brain by pushing the tissue into resected cortex or injecting it into deeper structures using thin metal cannula. The results showed that the immature dopamine precursors in fetal VM could survive the transplantation procedure while also maintaining their capacity for terminal differentiation and growth into functional mDA neurons.

Refinement of the procedure has been based largely on improving the survival and integration of dopamine neurons after transplantation while minimizing damage to the host. Important developments include the cell suspension technique that allowed the VM tissue pieces to be dissociated into more or less single cell suspensions prior to transplantation [4, 5] and a micro-transplantation approach using fine glass capillaries to deposit the suspension at precise locations with minimal damage to the host [6]. Cell suspension preparations allow for greater flexibility and reproducibility in transplantation procedures. Fixed volumes at a predetermined cell density can be distributed over multiple graft sites and across multiple animals using the same cell preparation.

Success in animal models of PD lead to the first clinical trials in patients in the 1980s. While the approach has been very successful for some patients [7], the overall variability in the therapeutic outcome [8] highlights the need for optimization of the approach through on-going experimental work in animal models of PD. A deeper understanding of the principles underlying successful restoration of motor function following engraftment of fetal mDA neurons—including survival, growth, and functional connectivity—also forms an important basis for the development of stem cell-based approaches for cell therapy in PD.

In this chapter, we describe the basic procedures for preparation of a VM cell suspension, as illustrated using E12.5 mouse. The dissection procedures are applicable to other species and for other donor ages and, notably, recent studies have suggested younger donor ages may result in better yields of dopamine neurons relative to the conventional choice of E12.5 for mouse and E14.5 for rat [9]. We also describe procedures for lesioning of the nigrostriatal pathway using the monoamine selective neurotoxin 6-hydroxydopamine, implantation of the cell suspension, and assessment of functional impact based on rotational behavior. These procedures are applicable also when using other sources of dopamine neurons for transplantation, such as stem cells.

For recommended further reading on fundamental principles and practical aspects of neural transplantation *see* refs. 10, 11.

4 Notes

1. The dissection microscope should allow suffi cient working distance between the stage and objective for manipulation of the embryos. It is helpful to use a backlit (beneath the stage) system with an adjustable mirror to give a range of contrast options. The addition of a halogen lamp and optical filters for fluorescence is particularly useful when working with transgenic mice expressing fluorescent proteins.
2. We routinely use L15 medium (Invitrogen), although any physiological salt solution may be acceptable—e.g., HBSS, DMEM, PBS, NaCl. Generally, quick dissections (i.e., <20 min) may be done under minimalist conditions (e.g., PBS, NaCl), while longer dissections are more likely to benefit from media containing nutritional supplements including salts and amino acids to support cell viability.
3. Grafts of ventral mesencephalon will invariably contain a fraction of serotonergic neurons originating from the developing raphe nucleus, along the ventral midline immediately caudal to the mid-hindbrain border. The position of the caudal dissection of the mesencephalic tube will thus impact on the amount of serotonergic progenitors included in the dissected tissue piece. This is relevant to mention in light of recent studies

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suggesting that high numbers of serotonergic neurons in striatal VM grafts may contribute unwanted side effects such as dyskinesias [12, 13]. Positioning the caudal dissection forward of the mid-hindbrain border will limit the contribution of serotonergic progenitors in the final cell preparation.

4. Move the dissected VM pieces away from the stage light and into a darker area towards the side of the petri dish. When dissecting large numbers of embryos, periodically move the dissected VM pieces to an eppendorf containing ice-cold cell culture medium (L15, HBSS, DMEM, or equivalent).
5. The meningeal tissue can be difficult to remove at earlier embryonic ages (i.e., <E13.5 for rat and <E11.5 for mouse). At these earlier ages, it is useful to incubate the dissected VM tissue piece(s) with collagenase/dispase (700 mg/ml; Roche) for 10 min at 37 °C before attempting to remove the meninges.
6. Pre-warm the incubation medium to 37 °C. Various cell culture media not containing Mg^{2+} or Ca^{2+} are available (e.g., $HBSS^{-Ca2+/-Mg2+}$, $DMEM^{-Ca2+/-Mg2+}$, $PBS^{-Ca\ 2+/-Mg2+}$) and may be acceptable. We routinely use $DMEM^{-Ca2+/-Mg2+}$.
7. Add at least 10–20 µl of medium per VM piece. Insufficient volumes will result in clumping of the cells and present difficulties when preparing the fi nal suspension for grafting.
8. We typically resuspend at 1×10^5 cells/µl in a PCR tube or small eppendorf. Using smaller tubes will reduce the risk of breaking the glass cannula when loading the cells (step 24). We have found densities > 1.5×10^5 cells/µl to occasionally be problematic during transplantation due to cell clumping and blockage of the glass injection cannula. For viability of the cell suspension, we have also found it best to work with larger volumes (>10) at the expense of cell density— i.e., in order to transplant 1×10^5 cells, consider preparing 10 µl at 0.5×10^5 cells/µl, and injecting 2 µl rather than 5 µl at 1×10^5 cells/µl and injecting 1 µl.
9. 6OHDA is light-sensitive and prone to oxidation. To minimize exposure to light and moisture, it is useful to aliquot stock 6OHDA into eppendorf tubes and note the amount of 6OHDA on the tube lid (e.g., 1–3 mg). Store aliquots in the dark at −20 °C. Change working solutions of 6OHDA every 2 h (ongoing oxidation of the toxin in solution is evident as a progressively deeper orange/brown color). The 6OHDA is generally supplied as a salt derivative (e.g., only 82 % free-base when supplied as 6OHDA hydrobromide, Sigma-Aldrich) and this should be accounted for when calculating the final dilution. The biological efficacy of 6OHDA stocks can show significant batch variation. When working with a new stock, it is recommended to test efficacy in a small group of animals (based on rotational behavior and/or immunohistochemistry for tyrosine hydroxylase) before undertaking large-scale experiments.
10. The dosage and placement of 6OHDA will depend on the aims of the experiment. Optimal stereotaxic coordinates may vary with the strain, sex, and age of the animal and should be determined by the user. For adult *rats*, complete lesioning of the nigrostriatal system (substantia nigral (SN) and ventral tegmental (VTA) dopamine neurons) can be achieved by injection of 14 µg of 6OHDA (4 µl of a 3.5 µg/µl solution) into the medial forebrain bundle of adult rats at the following stereotaxic coordinates: 4.4 mm caudal to bregma on the anterior–posterior axis (AP −4.4); 1.2 mm lateral to the midline (ML ±1.2); 7.8 mm below the dural surface (DV −7.8). Partial lesions (largely sparing the VTA neurons) can be achieved by multiple 6OHDA injections (2 µl of a 3.5 µg/µl solution per site) into striatal target areas of the SN neurons (AP +1/−0.1/−1.2; ML ±3/3.7/4.5; DV −5). For adult mice, lesioning of nigrostriatal dopamine pathway can be achieved through injection of 6OHDA directly into the midbrain (AP −3.0, ML ±1.2, DV −4.5) and the severity of the lesioning can be modulated by the dose of 6OHDA—e.g., 1.5 µl of a 1.5 µg/µl solution for partial lesioning and 2 µl of a 3 µg/µl solution for more complete lesioning. The results can vary markedly in mice and optimal dosage should be determined by the user. Mortality in mice (up to 50 %) can become an issue at larger doses for complete lesioning of the midbrain dopamine neurons.
11. The use of fine glass cannulas is recommended for intracranial injection procedures in rodents as a means to reduce physical damage and reflux of the injected material. Standard borosilicate glass capillaries (Harvard Instruments) can be pulled into fine glass cannula using a micropipette puller. The capillary can then be fitted to the end of a micro-volume syringe using heat-shrink tubing (Fig. 2). Before injecting the toxin, fill the microsyringe and attached glass cannula with vehicle (sterile 0.9 % saline) using a standard syringe to inject the microsyringe from the top before replacing the plunger. Use the plunger to draw up an air-bubble of 0.5–1 µl before drawing up the required amount of toxin from a small eppendorf. This will prevent dilution of the toxin by diffusion through the vehicle.

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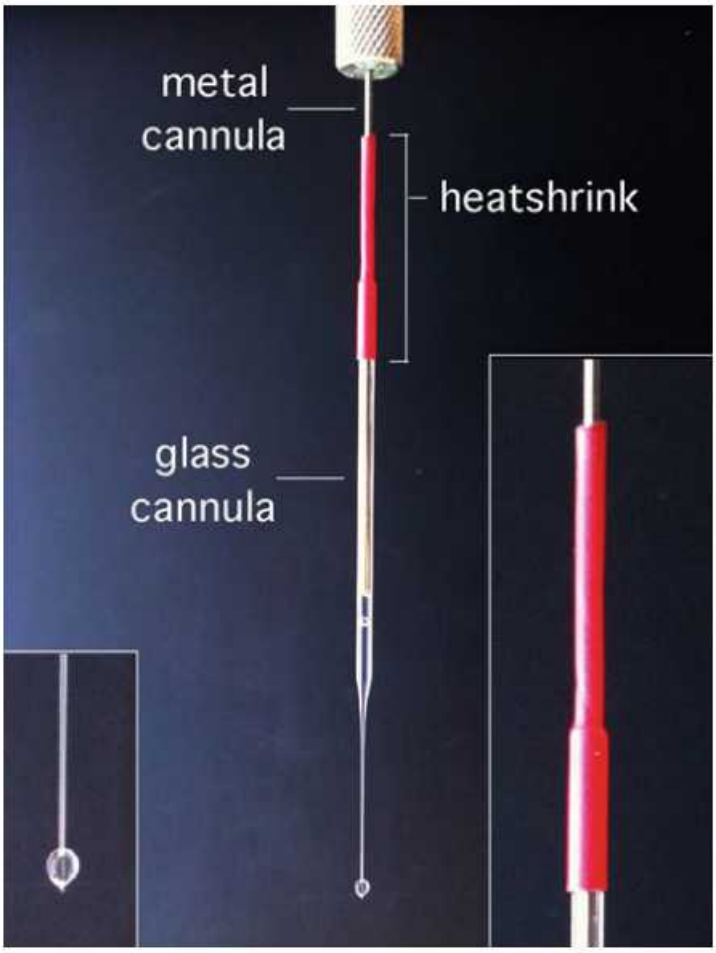

Fig. 2 Glass injection cannula fixed to a micro-volume syringe. The glass cannula can be fitted over the metal cannula of a micro-syringe using a sleeve of heat shrink (red). The sleeve is heated (e.g., using a handheld blowtorch) to form a water-tight seal between the glass and metal cannula. It is important to flush the syringe thoroughly with vehicle to ensure no leakage from either end of the heat-shrink seal before proceeding. The enlarged sections show the seal as well as a drop of saline at the tip of the glass cannula in greater detail. Note that it is also possible to use appropriately sized polyethylene tubing to join the glass and metal cannulas. In that case, the polyethylene is first fitted to the metal cannula and the glass is passed over the polyethylene tubing. It is normal to shear the polyethylene tubing to form a water-tight seal

12. 6-hydroxydopamine selectively destroys catecholaminergic neurons, including both dopamine and noradrenergic neurons. Although the ablation of noradrenergic neurons is largely overlooked in 6OHDA procedures targeting the nigrostriatal dopamine system, it may be important to protect the integrity of the noradrenergic system in certain cases depending on the specific aims of the experiment.
13. For apomorphine-induced rotation, all animals should be treated at least twice to allow for sensitization of the rotational response before recording rotational data on the third administration. It is recommended to allow a 48 h rest period between treatments. This is only required at the beginning of the experiment and subsequent rotational measurements in long-term experiments will only require a single apomorohine treatment.
14. Rotational behavior is most conveniently assessed using automated “rotometer” systems (e.g., Accuscan Instruments) whereby a harness placed around the animal is connected to a transducer that records the number and direction of rotations over time. Alternatively, rotations can be scored manually under suitable single-housing conditions, e.g., home cage or a large cylinder.
15. Unilateral lesioning of the nigrostriatal system will result in turning behavior towards the side of the lesion following amphetamine and away from the lesioned hemisphere following apomorphine. It is advisable to set a threshold response level of ≥6 turns per minute as an inclusion criterion when selecting animals for further experimentation. Lack of stability of the rotation response over time may become an issue when using animals with lower rotation scores. When determining animal numbers for experimental grouping, it should also be noted that there is invariably a population of “nonresponders” (up to 50 % depending on and species and strain). This aspect can be particularly problematic when using mice. A number of previous studies have provided detailed descriptions of the impact of 6OHDA lesioning on the nigrostriatal dopamine system and motor behavior in rats [14, 15] and mice [16, 17].

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16. Optimal stereotaxic coordinates should be determined by the user. As a guide, intra-striatalplacement can be achieved at the following coordinates: adult rats (AP +0.5, ML ±3, DV −4.6); adult mice (AP +1.0, ML ±2.3, DV −3.2). Grafting into the substantia nigra can be achieved using the following coordinates: adult rats (AP −5.3, ML ±2.2, DV −7.2); adult mice (AP −3.2, ML ±1.4, DV −4.2).
17. Backflow of single cell suspensions is an important but often overlooked issue in neural transplantation procedures. One should carefully monitor the point at which the cannula enters the brain to ensure there is no significant backflow of the suspension up the cannula and onto the surface of the brain. Ensuring the tip of the cannula is “flat” (rather than jagged and broken) may help to prevent backflow. This can be achieved by scoring and snapping the end of the cannula. If backflow presents as a consistent problem during surgery, it is recommended to replace the cannula.

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MATERIALS TEXT

2.1 Preparation of Cell Suspensions

2.1.1 Apparatus

1. Dissection microscope (See Note 1).
2. Dissection instruments including micro-scissors (e.g., spring/ vannas, 5–8 mm blades) and Dumont forceps (World Precision Instruments).
3. Hemocytometer and coverslips.
4. Light microscope for basic cell counting.
5. Benchtop centrifuge (preferably refrigerated).

2.1.2 Reagents

1. Cell media including: L15 (Leibovitz) and DMEM$^{-Ca2+/-Mg2+}$, HBSS$^{-Ca2+/-Mg2+}$, PBS$^{-Ca2+/-Mg2+}$ (Invitrogen) or similar.
2. DNase I (Roche). Reconstitute lyophilized protein with dH_2O to a final concentration of 10 mg/ml. Aliquot and store at −20 °C. Dilute 1:200 in medium$^{-Ca2+/-Mg2+}$ for a 0.05 % working solution.
3. Trypsin (Invitrogen). Aliquot 0.25 or 2.5 % stock and store at −20 °C. Dilute ×2.5 or ×25 in medium$^{-Ca2+/-Mg2+}$ for a working solution of 0.1 %.
4. Plastic or glass Petri dish.
5. Plastic Eppendorf tubes.
6. Trypan blue (Invitrogen). For some microscopes with lower light intensity, it may be necessary to dilute the stock Trypan blue 1:10 in PBS in order to distinguish viable (dye-excluding) from dead cells.

2.2 Surgical Procedures and Rotational Behavior

2.1.2 Apparatus

1. Stereotaxic frame with appropriate adaptors for fixed-skull positioning of mice and/or rats (e.g., Stoelting, Kopf, Harvard Instruments).
2. Large probe holder for syringe (Stoelting).
3. Micro-volume syringe with plunger and metal cannula (5–10 µl, e.g., Hamilton or SGE Analytical Science).
4. Surgical microscope, preferably with internal light-source.
5. Suitable handheld drill with appropriate sized drill bit (~1–3 mm).
6. Micropipette puller.

2.2.2 Reagents

1. Borosilicate glass capillaries (Harvard Instruments). Note that the appropriate internal diameter will depend on the outer diameter of the metal cannula on the microsyringe.
2. Heat-shrink tubing, 1.2–1.5 mm (electrical supplies, e.g., RadioParts).
3. 6-hydroxydopamine hydrobromide (6OHDA; Sigma).
4. 0.9% Sterile saline.
5. Ascorbic acid (Sigma).
6. Desipramine (optional; Sigma).
7. Lidocaine (optional; scheduled pharmaceutical).
8. Temgesic (optional; scheduled pharmaceutical).
9. D-Amphetamine (scheduled pharmaceutical).
10. Apomorphine (scheduled pharmaceutical).

SAFETY WARNINGS

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

BEFORE STARTING

All procedures should be performed under aseptic conditions.

3.1 Preparation of Cell Suspensions

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1 Remove the uterus immediately from euthanized or terminally anesthetized animals via caesarian section, place in cold L15 medium (*see* **Note 2**) and store **On ice** .

2 Place the embryos in a petri dish containing **ice-cold** L15 medium. Under a dissection microscope (*see* **Note 1**), remove each embryo using suitably fine dissection instruments. This is best achieved through a single incision parallel to the placental mass (*see* Fig. 1a, b) while applying gentle pressure with the forceps to both sides of the sac—the positive pressure in the amniotic sac will usually expel the embryo. Cut the umbilical cord (Fig. 1c) and repeat for the next embryo.

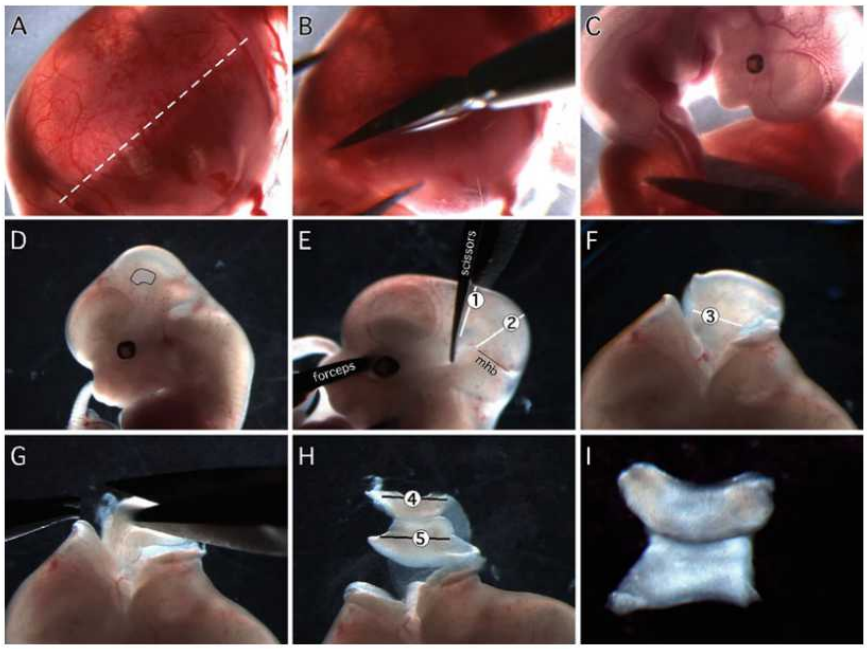

Fig. 1 Dissection of ventral mesencephalon from E12.5 mouse. (**a**) The placental mass can be seen through the intact embryonic sac using a dissection microscope with a backlit stage. The *dashed line* illustrates a convenient location to make an incision (**b**) using micro-scissors and forceps. (**c**) The umbilical cord is cut once the embryo is expelled from the embryonic sac. (**d**) The grayed-out region highlights the approximate area of the ventral part of the neural tube as the target for dissection. (**e**) The white lines indicate the anterior (*1*) and posterior (*2*) dissection planes for isolating the midbrain from the embryo. The mid-hindbrain border (mhb) is indicated in black . (**f**) The connective meningeal tissue can be separated from the underlying VM through an incision perpendicular to the first two cuts (*3*) by sliding a blade in between these tissue layers (**g**). Rolling the now isolated midbrain tube 90° away from the user will expose the ventral floor plate (**h**). The ventral part of the midbrain can now be isolated by two parallel cuts as indicated by the black lines (*4, 5*). (**i**) The final dissected VM piece has a characteristic “butterfly” shape

3 The ventral mesencephalon can be obtained from each embryo in five steps (step 4 - step 8) using forceps to stabilize the embryo and scissors to isolate the VM as a single piece of tissue (Fig. 1):

4 (a) The anterior end of the mesencephalic tube is cut at approximately the di-mesencephalic boundary—parallel and directly adjacent to where the narrow tube expands into the larger forebrain regions containing the developing cortex and thalamus (Fig. 1e).

5 (b) The caudal end of the mesencephalic tube is cut at approximately the level of the mid-hindbrain boundary (Fig. 1e; *see* **Note 3**).

6 (c) The mesencephalic tube can now be isolated by sliding one blade of the scissors between the meningeal layers and the VM—perpendicular to the cuts performed in **step 4 and step 5** (a, b)—in order to cut the connective tissue overlaying the midbrain (Fig. 1f, g).

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7 (d) The ventral midline is exposed by “rolling” the VM tube 90° away from the user (Fig. 1h). The dorsal midline will remain attached to the meningeal tissue, face-down on the petri dish. Cut parallel to the midline approximately 1/3 of the distance between the ventral and dorsal midline on one side of the VM tube (Fig. 1h).

8 (e) Perform a second cut at the same position on the other side of the tube to free the final VM piece used for transplantation (Fig. 1h; *see* **Note 4**). It may be necessary to trim excess tissue and it is important to peel away any remaining meningeal tissue (*see* **Note 5**).

9 Remove the media from the eppendorf containing the pooled VM pieces and replace with medium that does not contain Ca^{2+} or Mg^{2+} (e.g., DMEM/PBS/HBSS$^{-Ca\ 2+/-Mg2+}$) and is supplemented with **0.05 % DNase** and **0.1 % trypsin** . Incubate at **37 °C** for **00:20:00** (*see* **Note 6**). 20m

10 Rinse the VM pieces in medium$^{-Ca2+/-Mg2+}$ three times before adding a known volume of medium$^{-Ca2+/-Mg2+}$ with **0.05 % DNase** for tissue dissociation (*see* **Note 7**).

10.1 Rinse the VM pieces in medium$^{-Ca2+/-Mg2+}$.

10.2 Rinse the VM pieces in medium$^{-Ca2+/-Mg2+}$.

10.3 Rinse the VM pieces in medium$^{-Ca2+/-Mg2+}$.

10.4 Add a known volume of medium$^{-Ca2+/-Mg2+}$ with **0.05 % DNase** for tissue dissociation.

11 Mechanically dissociate the VM tissue pieces by gently trituration with a 1 ml pipette tip followed by a 200 µl tip. Continue as necessary to achieve a cloudy suspension.

12 To estimate the total viable cell number, dilute a small (e.g., **1 µl** – **5 µl**) sample of the suspension 1:10 in Trypan blue and count viable (dye-excluding) cells using a hemocytometer. Total viable cells = cells counted × dilution factors (e.g., trypan dilution and fractional area of hemocytometer counted) × volume of suspension. Cell viability should be >90%.

13 Centrifuge at **500 x g, 4°C, 00:05:00** . Remove the supernatant and use a pipette to resuspend the pellet in medium$^{-Ca2+/-Mg2+}$ with **0.05 % DNase** at the desired cell density (*see* **Note 8**). Be careful not to introduce air bubbles when resuspending. Store **On ice** throughout transplantation procedure. 5m

3.2 Unilateral Lesioning of the Nigrostriatal Dopamine System in Rodents

14 Prepare the 6-hydroxydopamine (6OHDA) solution by dissolving 6OHDA in sterile saline (**0.9 %**) containing

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[M]**0.02 mg/ml L-ascorbic acid (Sigma)** to achieve the desired concentration (*see***Note 9**).

Do not use PBS, this will oxidize the toxin (*see***Note 9**).

15 Rodents should be deeply anesthetized with the head placed in a fixed-skull position using a stereotaxic frame.

16 Make an incision in the scalp along the midline and drill a small burr hole in the skull at the desired coordinates (*see* **Note 10**) to reveal the dural surface.

17 Inject the 6OHDA solution using a micro-volume syringe, preferably fitted with a pulled glass cannula (*see***Note 11**). 2m
Leave the cannula in place for at least **00:02:00** after injection to allow for diffusion of the toxin. If necessary, the central noradrenergic system can be protected by intraperitoneal injection of desipramine ([M]**20 mg/kg** ; Sigma) 30 min before injection of 6OHDA (*see***Note 12**).

18 Close the scalp (e.g., using suture or Michel clips). A topical anesthetic (e.g., lidocaine) and/or a central analgesic (e.g., Temgesic 0.3 mg/kg) may be administered. This is particularly relevant for short recovery times, e.g., when using inhaled anesthesia.
The functional impact of unilateral lesioning of the nigrostriatal pathway can be readily determined by measuring turning behavior in response to D-amphetamine or apomorphin.

19 For D-amphetamine-induced rotation, dissolve D-amphetamine in [M]**0.9 % sterile saline** and administer (i.p.) [M]**2.5 mg/kg** for rats or [M]**5 mg/kg** for mice. For apomorphine-induced rotation, dissolve apomorphine in [M]**0.9 % saline** with [M]**0.02 mg/ml L-ascorbic acid** administer (s.c.) [M]**0.025 mg/kg** for rats or [M]**0.1 mg/kg** for mice (*see***Note 13**).

20 Allow the animals to habituate for **00:10:00** after drug injection. Record the number of full (360°) body rotations in 1h 40m each direction over a defined time period—typically **00:40:00** – **01:00:00** for apomorphine and **01:00:00** – **01:30:00** for amphetamine (*see***Note 14**).

21 Record the rotation score for each animal as net full body rotations per minute in one direction (*see***Note 15**).

3.3 Implantation of Cell Suspension 2m

22 Prepare deeply anesthetized animals in a sterotaxic frame and drill a burr hole through the skull at the desired coordinates as per steps 15 and 16 (*see***Note 16**).

23 Resuspend the cells through gentle titruation using a pipette to achieve a uniform suspension.

It is important to repeat this step prior to each implantation as an effort to deliver a consistent number of cells across all animals.

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24 Prefill the microsyringe and glass cannula with vehicle (e.g., [M]**0.9 % saline** or cell medium) and draw the required volume of cells into the glass cannula through the tip (unlike when using aqueous solutions, there is no need to draw an air-bubble first).

25 Slowly pressure-inject the cells at the desired location. It is important to monitor cell-flow through the glass cannula[2m] throughout the injection to ensure that all the suspension has been injected (this may not be the case if there is a blockage or a leak in the cannula setup) and, importantly, that there is no backflow of the injected contents during the injection (*see* **Note 17**). Keep the cannula in place for at least **00:02:00** before slowly withdrawing.

26 Close the scalp and administer local anesthetic and central analgesia as per step 18 above.

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