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Dopamine neurons derived from embryonic stem cells efficiently induce behavioral recovery in a Parkinsonian rat model

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Abstract

To test the in vivo effect of a high yield of dopaminergic (DA) neurons (90% of total neurons) which had been generated from a genetically modified mouse embryonic stem cell line, N2, the cells were transplanted into a rat model of Parkinson's disease (PD). The PD animals grafted with N2-derived cells showed significant behavior improvements compared with sham controls from 2 weeks posttransplantation, whereas animals with naïve D3-derived cells (~28% DA neurons of total neurons) showed only a modest recovery. Furthermore, hyperactivity observed in the subthalamic nucleus, pedunculopontine nucleus, and substantia nigra pars reticulata of PD rat models was dramatically reduced by the grafting of N2-derived cells. The number of DA neurons in the striatum which originated from N2 grafting was much higher compared to that from D3 grafting, and the neurons efficiently released DA in the brain, showing a good correlation with behavioral recovery.

Keywords: ES cells; Differentiation; Dopamine neurons; Parkinson's disease; Behavior recovery

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the progressive loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and a reduction in striatal dopamine [1,2]. In PD, the loss of DA neurons in the SNpc leads to impaired information processing in the basal ganglia. More specifically, it is thought that the reduction in the dopamine level is responsible for the imbalance in the activities of the direct and indirect pathways from the striatum to the basal ganglia output structures, the pars reticulata of the substantia nigra (SNpr), and the internal portion of the globus pallidus (GPi) [3,4]. The increased activity of basal ganglia output structures in the dopamine-depleted state may be partially due to an elevation of the excitatory drive from the subthalamic nucleus (STN). The reduction of subthalamic neuronal output has been found to reverse the behavioral effects in Parkinsonian rats, primates, and humans [5–10].

Although neurosurgical treatment has been found to be beneficial for some patients with advanced PD, the prevailing strategy for the treatment of PD is pharmacological. However, pharmacological treatment with L-DOPA has some limitations because its effectiveness decreases over time and side effects develop [11]. Thus, an alternative approach for restoration of the damaged DA system is transplantation of DA-synthesizing cells. Many studies

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have reported that fetal brain cells can relieve the symptoms of PD [12,13]. However, this approach also has limitations, including an ethical issue and the technical difficulty of obtaining large amounts of fetal brain tissue [14–18].

To obtain DA-synthesizing cells from other sources, many scientists have tried to develop protocols for inducing DA neurons from ES cells [19–26]. We recently reported that, in the presence of signaling molecules, the co-culturing of Nurr1-expressing mouse ES cells (N2 or N5) with PA6 stromal cells can synergistically generate enough DA neurons to compose up to 90% of total neurons [27]. Here, we show that the high yield of DA neurons efficiently functions in a Parkinsonian rat model.

Materials and methods

Production of a Parkinsonian rat model, cell transplantation, and behavioral testing. Male Sprague–Dawley rats weighing 200–230 g were used to generate Parkinsonian rat models. The surgical procedure was performed as described previously [28,29].

After two weeks of the development of 6-OHDA-induced lesions, animals were tested for apomorphine-induced turning behavior (apomorphine at 0.1 mg/kg i.p. in saline containing ascorbic acid at 2 mg/ml; Sigma, St. Louis, MO, USA) and forepaw adjusting stepping, as described in a previous study [30].

One week after behavioral testing, ES cells were transplanted using a sterilized stainless steel needle (0.3 mm OD) connected to a Hamilton microsyringe. Undifferentiated mES cells (the wild-type D3 and Nurr1-expressing N2) were maintained and differentiated as described previously [27]. Briefly, they were maintained on gelatin-coated dishes in DMEM (Gibco, Rockville, MD) supplemented with 2 mM glutamine (Gibco), 0.001% 2-mercaptoethanol (Gibco), 1× non-essential amino acids (Gibco), 10% donor horse serum (Sigma), and 2000 U/ml human recombinant leukemia inhibitory factor (LIF; R&D Systems, Minneapolis, MN). To differentiate ES cells, PA6 cells were plated on gelatin-coated culture dishes to make a uniform feeder monolayer 1 day before the addition of D3 or N2 cells. ES cells were then allowed to differentiate on the PA6 feeder cells for 14 days. ES differentiation medium I [G-MEM medium (Gibco) supplemented with 10% knockout serum replacement (KSR, Gibco), 0.1 mM non-essential amino acids (Gibco), 1 mM sodium pyruvate (Sigma), 0.1 mM 2-mercaptoethanol (Gibco), and PEST (Gibco)] was used for 8 days and then replaced with ES differentiation medium II [G-MEM (Gibco) supplemented with N-2 supplement (Gibco), 0.1 mM non-essential amino acids (Gibco), 1 mM sodium pyruvate (Sigma), 0.1 mM 2-mercaptoethanol (Gibco), and PEST (Gibco)] for an additional 6 days. During differentiation of N2 cells, signaling molecules such as sonic hedgehog (Shh), fibroblast growth factor (FGF) 8 were treated during days 5–9 and ascorbic acid was added during days 9-14. Naïve D3 and N2-derived cells were transplanted into rat striatum at in vitro differentiation day 9 (precursor cells) of total 14 days. Four microliter of the cell suspension (1×10^5 cells/μl) was injected into the ipsilateral striatum [AP, +0.2 mm; ML, 3.0 mm; DV, 4.5 mm (2 μl) and 5.5 mm (2 μl), respectively] over a period of 3 min. A time lapse of 4 min before the removal of the needle allowed the cells to settle down. The rats were given an injection of cyclosporin A (10 mg/kg, i.p.; Chong Kun Dang Pharm., Seoul, Korea) 24 h before grafting and this was continued until sacrifice. Behavioral testing was conducted after the 2nd, 4th, 6th, and 8th weeks of transplantation.

Experimental groups were divided into 4 groups: (i) a normal group, 10 rats without lesions; (ii) a sham control group with 6-OHDA lesions ($n = 10$); (iii) a 6-OHDA lesioned group transplanted with neurons derived from wild-type mouse ES cells (D3, $n = 25$); and (iv) a 6-OHDA lesioned group transplanted with neurons derived from Nurr-expressing ES cells (N2, $n = 25$). Teratoma formation was observed in four animals among 50 animals for D3 and N2 cell grafts.

Extracellular single-unit microrecording and histological assessment for transplanted TH^+ cells. The extracellular unit recording procedure (normal rat: $n = 5$, PD model: $n = 5$, D3 graft: $n = 10$, and N2 graft: $n = 10$) and statistical analysis were performed as described previously [28].

Histological assessment for transplanted TH^+ cells (normal rat: $n = 4$, PD model: $n = 5$, D3 graft: $n = 16$, and N2 graft: $n = 17$) was performed by modifying the methods reported previously [25,31,32]. Briefly, 10 weeks after transplantation of D3 and N2 cells, the grafted rats were anesthetized with 25% urethane (Sigma) in PBS and intracardially perfused with 125 ml of normal saline followed by 250 ml of ice-cold 4% paraformaldehyde in PBS. Brains were postfixed in the same solution, cryoprotected with 30% sucrose in PBS for 48 h, and frozen. Brains were sectioned on a freezing microtome (section thickness: 10 μm) and collected in PBS. Serial sections spanning the graft were made. The staining of TH^+ neurons was performed using rabbit anti-TH antibody (Pel-Freeze Rogers, AK, USA, 1:250). Counting of TH^+ neurons was performed on every tenth of serial sections using a Zeiss Axioplan light microscope with a 20× lens. Quantitative data from serial sections were expressed as means ± SEM/section.

Analysis of dopamine. For DA detection in vivo, animals in each group (normal rat: $n = 3$, PD model: $n = 3$, D3 graft: $n = 4$, and N2 graft: $n = 4$) were implanted with microdialysis probes (CMA/11 Guide Cannula, CMA Microdialysis AB, Solna, Sweden) in the ipsilateral striatum (coordinates: AP to bregma, +1.2 mm; LA from midline, 2.5 mm; ventral to the surface of the dura, −6.0 mm). The probe was perfused with artificial cerebrospinal fluid (aCSF) at a flow rate of 2 μl/min for 4 h (CMA102 Microdialysis Pump). The Guide cell (+400 mV) was used to perform a partial clean-up of the mobile phase prior to sample injection. The samples were introduced into the autosampler at a mobile phase flow rate of 1.0 ml/min. Neurotransmitter separation was carried out by means of a reverse-phase column (ESA HR-80 column: 3 μm, ODS, 80 × 4.6 mm; ESA Bioscience, Cheimsford, MA, USA). Typical values of applied potential used in the present experiment were −100 mV at Electrode 1 and +200 mV at Electrode 2. Chromatograms were analyzed with peak areas, which were classified with the retention times of reference substances.

Results

High yield of DA neurons from mouse ES cells efficiently induces behavior recovery in a Parkinsonian rat model

We recently showed that the co-culturing of Nurr1-expressing mouse ES cells (N2) with PA6 stromal cells synergistically generates a high proportion of DA neurons up to 90% of total neurons, while naïve D3 cells induce a composition of about 28% DA neurons [27]. These neurons expressed high levels of midbrain DA markers and released DA. Furthermore, the majority of generated neurons exhibited electrophysiological properties characteristic of DA neurons. To examine the in vivo effect of the DA neurons, the cells were transplanted into an animal model of PD.

The PD models induced by the 6-OHDA were confirmed by the observed increase in the apomorphine-induced rotation and the observed reduction in the forepaw stepping number (Pre-TP in Figs. 1A and B). The extent and location of the lesions were also confirmed by assessing the loss of TH-immunoreactive cells and fibers in the SNpc and striatum. After 3 weeks of lesion development, animals were given a suspension of 4×10^5 ES cells into the striatum. We transplanted the cells into the striatum of rat PD models on the 9th day of a 14 day in vitro differentiation. Effects of the grafted cells on behavior recovery were

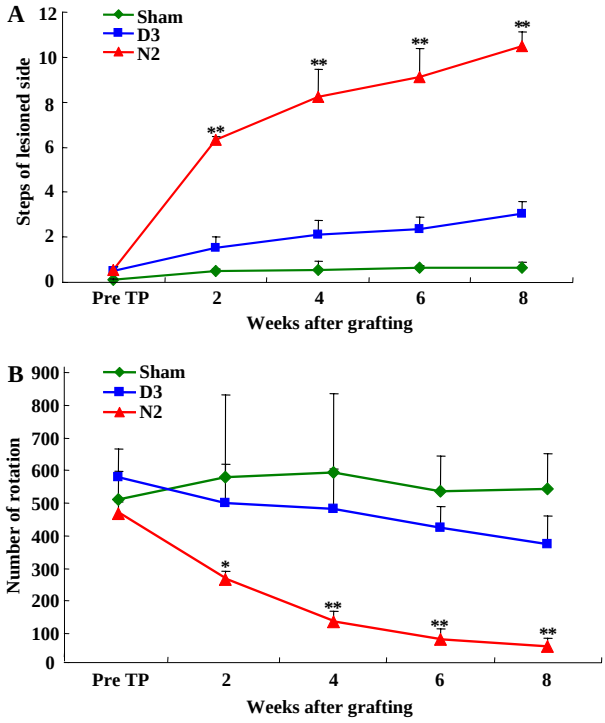

Fig. 1. Behavioral effects of grafted ES cells. The behavior in PD animals grafted with neurons derived from wild-type D3 cells ($n = 25$) or Nurr1-overexpressing N2 cells ($n = 25$) and sham controls ($n = 10$) was tested before transplantation (pre TP) and at 2, 4, 6, and 8 weeks postgrafting. (A) The results in the adjusting step test are expressed as the number of steps/0.9 m of treadmill (at a rate of 0.075 m/s) with the lesioned side forepaw. Animals grafted with N2 cells showed a dramatic increase (10.49 ± 0.64 at 8 weeks) compared to sham controls (0.63 ± 0.275 at 8 weeks). Animals with D3 cells showed modest, but non-significant, increase. (B) Apomorphine-induced rotation response per hour. Animals with sham surgery showed no change in rotational score over time (545 ± 105.6 at 8 weeks). In contrast, animals with N2 cells showed a significant reduction in rotation over time (60.64 ± 24.2 at 8 weeks). Animals with D3 cells showed modest, but non-significant, reduction. * Significantly different from sham controls at $p < 0.05$, $^{**}p < 0.01$.

evaluated at the 2nd, 4th, 6th, and 8th weeks of transplantation with forepaw adjusting step and an apomorphine-induced rotation test (Fig. 1).

The forepaw adjusting step is a non-pharmacological test and thus reflects a more direct measure of motor deficits [30]. As shown in Fig. 1A, a control PD group (sham) showed almost no stepping until 8 weeks. However, the PD animals grafted with differentiated N2 cells (90% DA neurons/total neurons) showed a significant improvement over time in stepping number and recovery of >75% (>10 steps) at 8 weeks after transplantation as compared with the wild-type (~13 steps, data not shown). In contrast, the PD models grafted with naïve D3-derived cells (28% DA neurons/total neurons) showed a modest improvement in stepping over time, about 3 steps at 8 weeks postgrafting. We next tested behavior recovery by apomorphine injection (Fig. 1B). Apomorphine stimulation of the PD models induces a movement bias contralateral to the lesion side. Sham controls showed a consistently high number of rotations (>500), whereas the PD models grafted with N2-derived cells showed a significant reduction in rotational scores from 2 weeks posttransplantation compared with a control group (sham surgery). As in a forepaw adjusting step test (Fig. 1A), however, animals grafted with D3-derived cells showed a slight, but not significant, decrease in rotation compared with the control sham group. Graft sizes after transplantation of D3- and N2-derived cells were similar (data not shown). Taken together, high levels of DA neurons differentiated from N2 ES cells more efficiently bring about behavior recovery in a Parkinsonian rat model than did wild-type D3 ES cell-derived neurons.

Electrophysiological effects and DA production of grafted ES cells

To electrophysiologically investigate the transplantation effect, we examined the firing rates and firing patterns in the PPN, SNpr, and STN by extracellular single-unit microelectrode recording. In PD, the progressive deficit of DA cells in the SNpc results in hyperactivity in the SNpr, STN, and PPN due to impaired information processing in the basal ganglia [3,5,6]. As previously reported [28], we observed hyperactivity in the SNpr, STN, and PPN of 6-OHDA lesioned rats (Fig. 2) [33,34]. PD models with sham surgery exhibited a significant increase in the mean firing rates of three areas compared to normal rats (Fig. 2A). Following ES cell grafting in the PD models, however, the mean firing rates were significantly decreased in all three areas compared with sham controls 10 weeks postgrafting. The firing rates in PPN and SNpr were slightly lower in N2 grafts than in D3 grafts, but the firing rate in STN was almost the same between the two grafts. However, the reason the firing rates in the grafts became lower than those in normal rats is yet to be elucidated. Representative firing patterns in the SNpr of each group are shown in Fig. 2B. Neuronal activity of a sham control was higher (27.8 spikes/second) compared to that in a normal rat (20.8 spikes/second). In hemi-Parkinsonian models grafted with DA neurons derived from N2 cells, the firing activity was significantly decreased (10.4 spikes/second). These results demonstrate that reinnervation of DA neurons by the grafting of ES cells induces decreased firing rates in SNpr, STN, and PPN neurons. Each position of extracellular single-unit microelectrode recording was confirmed by the identification of neurons by their stereotaxic location and the histological location of the electrode tip after iontophoresis with pontamine sky blue (−18 μA for 20 min) (Fig. 2C).

To examine the correlation between the behavior recovery and the number of DA neurons in the graft, we next analyzed the TH^+ cells which originated from ES cells in the striatum of the rat brain (Figs. 3A and B). Ten weeks after transplantation, the recipient rats were sacrificed, fixed by perfusion, and then analyzed for TH^+ neurons. In the N2-derived cell grafts, we were able to find many TH^+ cell bodies (Fig. 3A, lower). In contrast, fewer TH^+

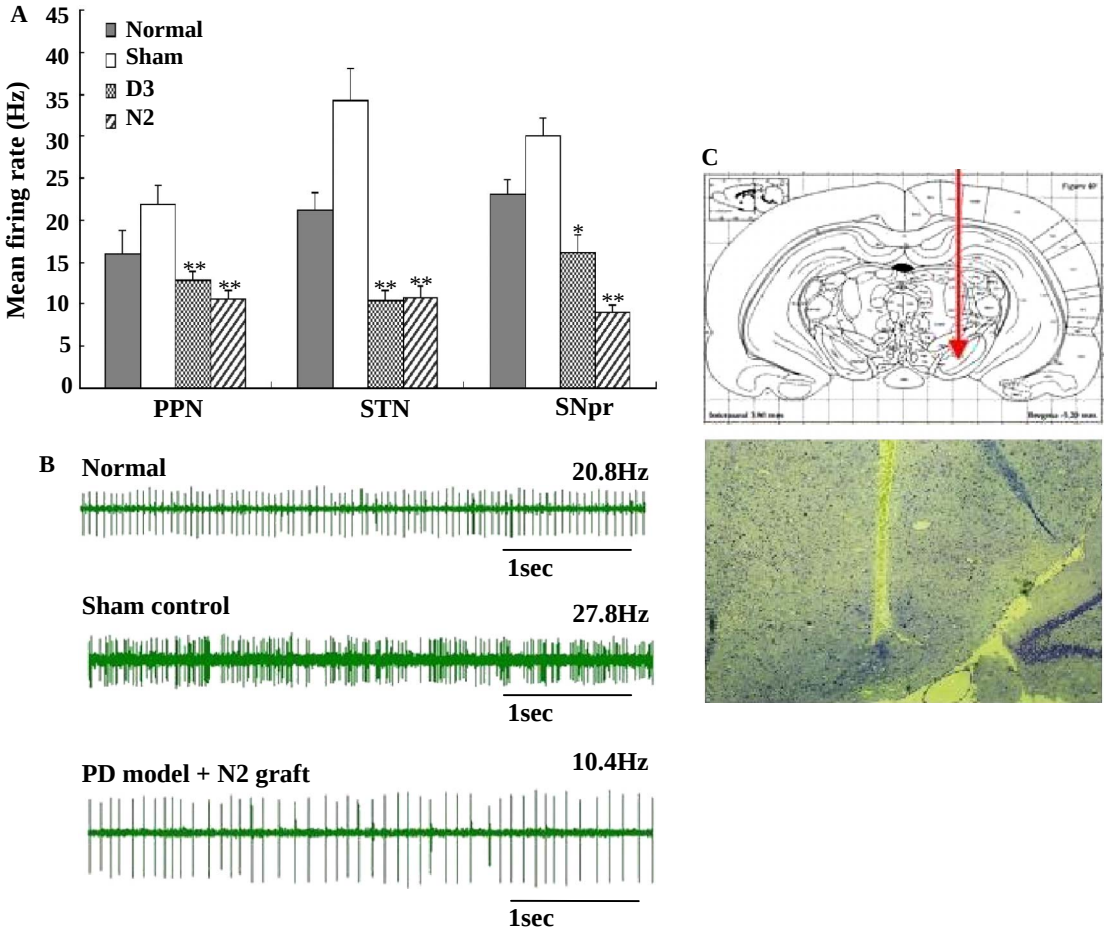

Fig. 2. In vivo electrophysiological effects of grafted ES cells. The extracellular single-unit recordings were performed from STN, SNpr, and PPN in hemi-Parkinsonian models grafted with DA neurons derived from D3 or N2 cells and in sham controls at 10 weeks postgrafting. (A) Compared to the normal rats, PD rat models exhibited a significant increase in mean firing rates in the SNpr, STN, and PPN. Following ES cell (D3 or N2) grafting in the PD models, the mean firing rates in the SNpr, STN, and PPN were significantly decreased. $^{*}p < 0.05$, $^{**}p < 0.01$ in comparison with values from sham controls. (B) Representative discharge patterns of SNpr recorded in each group. (C) Photomicrograph showing the pontamine sky blue mark corresponding to a neuron which was recorded at the end of a track in SNpr. Magnification, 40×.

cell bodies were detected in the D3-derived cell grafts (Fig. 3A, upper), although the same number of cells was transplanted in both cases. When we counted the number of TH^+ cells (Fig. 3B), we determined the TH^+ cells/section to be 187.63 ± 18.7 for the N2 graft and 52.13 ± 5.33 for the D3 graft. The difference in the number of TH^+ cells is consistent with the difference in behavior recovery between N2 and D3 grafts, indicating a good correlation between TH^+ cell numbers and behavior recovery. The DA level in the striatum was analyzed by microdialysis (Fig. 3C). The DA level in PD models was much lower than that in normal rats (data not shown), whereas the DA level (~0.08 μM) in animals grafted with N2-derived cells was almost the same as that (~0.09 μM) of normal rats (Fig. 3C), showing that transplanted N2 cells efficiently release DA, and thus, induce functional recovery of brain circuitry. However, DA level (~0.05 μM) secreted by the D3-derived cell grafts was lower than that (~0.08 μM) by N2 cell grafts. Taken together, our results show that the high yield of DA neurons generated from mouse ES cells also efficiently functions in vivo after transplantation in a Parkinsonian rat model.

Discussion

The distinguishing features of ES cells are their capacity to be maintained indefinitely in an undifferentiated state and the ability to develop into multilineage cells under certain conditions. By overexpressing the Nurr1 transcription factor (N2 cell line) and coculturing with PA6 stromal cells in the presence of signaling molecules, such as SHH and FGF8, we have developed an in vitro protocol by which a high yield of DA neurons (90% of total neurons) was efficiently generated from mouse ES cells [27]. In this study, we attempted to transplant the DA neurons produced by this protocol into a Parkinsonian rat model to determine whether these neurons also efficiently bring about a functional improvement in PD animal models.

Behavior recovery by N2 cell grafting (~90% DA neurons/total neurons) was much more prominent in both

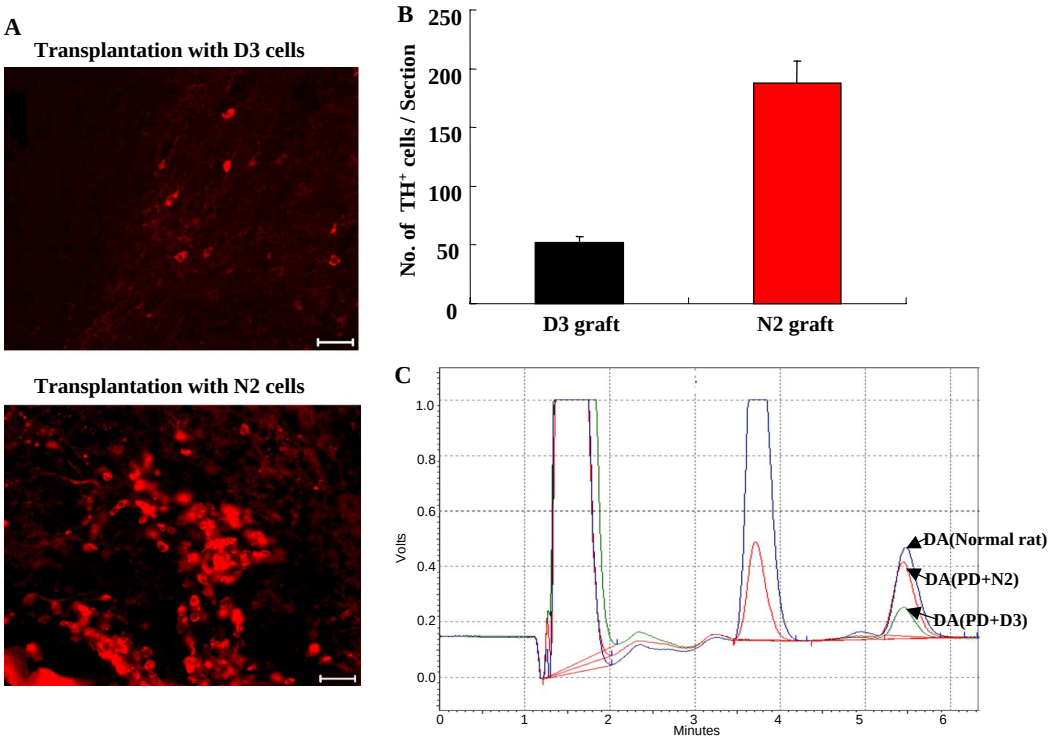

Fig. 3. N2 cells show good survival after grafting into the striatum of hemi-Parkinsonian rats. (A) Immunohistochemical staining using anti-TH antibody at 10 weeks after implantation of D3 and N2 ES cells into 6-OHDA lesioned striatum. Numerous TH^+ neurons were found within the N2 cell grafts (lower) in comparison to D3 cell grafts (upper). Scale bars, 50 μm. (B) Number of TH^+ neurons/section in the striatum at 10 weeks after transplantation (4×10^5 cells) with D3 and N2 ES cells. The number of DA neurons/section in N2 and D3 grafts was 187.63 ± 18.7 and 52.13 ± 5.33, respectively. (C) The amount of DA in the striatum was analyzed by microdialysis. DA level (~0.08 μM) secreted by the N2 ES cell graft, but not D3 cell graft (~0.05 μM), in a hemi-Parkinsonian striatum was similar to that (~0.09 μM) of a normal rat.

apomorphine-induced rotation and forepaw stepping tests compared to naïve D3 cell grafting (~28% DA neurons/total neurons) (Fig. 1). These results indicate that the efficient in vitro protocol can be translated to the in vivo context well.

In the PD model, hyperactivity in the SNpr, STN, and PPN can be induced by impaired information processing in the basal ganglia which results from the death of DA cells in the SNpc. A current model of the motor pathway postulates that D1 dopamine receptors on striatonigral neurons act to increase the activity of the inhibitory GABAergic striatonigral projection [35,36]. Thus, due to the DA loss of the striatum in rat PD models, striatal GABAergic neurons projecting to SNpr should become underactive, leading to less inhibition of this output nucleus. This, in turn, should lead to overactivation of the GABAergic SNpr neurons, which would be further amplified by an increased excitatory input from the STN [37]. The hyperactivity of the STN is based on the hypothesis that the loss of DA in the striatum causes a reduction in the activity of the inhibitory GABAergic pallidosubthalamic pathway [3]. Interestingly, hyperactivity in the SNpr, STN, and PPN of PD models was dramatically reduced by the transplantation of ES cell-derived neurons (Fig. 2), which signifies the restoration of neuronal circuitry. In PPN and SNpr, the firing rates were slightly lower in N2 grafting than in D3 grafting, although they were similar in STN.

Fig. 3 shows that, 10 weeks after transplantation, a higher number of DA neurons were detected in the striatum of N2-grafted animals compared with that of D3-grafted animals although graft sizes between two were similar. The number of DA neurons was 3.6-fold higher in N2-grafted animals than in D3-grafted animals (Fig. 3B). This difference in the number of DA neurons led to the difference in the behavior recovery of the N2- and D3-grafted animals (Fig. 1). Although the DA content in PD models with D3-derived cells was low (~0.05 μM), the DA content (~0.08 μM) secreted from PD animals with N2-derived cells was similar to that (~0.09 μM) of the normal rat (Fig. 3C), and the level of behavior recovery in the PD model with N2-derived cells nearly reached the level of normal rats by 8 weeks after grafting (Fig. 1). Thus, this DA secretion level correlates well with the degree of behavior recovery. Therefore, these results suggest that our in vitro protocol is also very effective in the in vivo context after transplantation. The reinnervation of DA neurons by the transplantation of ES cell-derived neurons is believed to improve Parkinsonian motor symptoms, presumably by reducing the activity of the basal ganglia output structures [30,38]. The observed behavioral effects were dependent on the survival of DA neurons within the striatum, since graft-

ing of other tissue produces no behavioral effects [39,40]. Following the development of an efficient differentiation method from mouse ES cells and application of these cells to a Parkinsonian rat model, in the next step, we are now pursuing the efficient induction of DA neurons from human ES cells and their application to Parkinsonian animal models.

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