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Transplantation of subventricular zone neural precursors induces an endogenous precursor cell response in a rat model of Parkinson's disease

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Abstract

Realistically, future stem cell therapies for neurological conditions including Parkinson's disease (PD) will most probably entail combination treatment strategies, involving both the stimulation of endogenous cells and transplantation. Therefore, this study investigates these two modes of neural precursor cell (NPC) therapy in concert in order to determine their interrelationships in a rat PD model. Human placental alkaline phosphatase (hPAP) labeled NPCs were transplanted unilaterally into host rats which were subsequently infused ipsilaterally with 6-hydroxydopamine (6-OHDA). The reaction of host NPCs to the transplantation and 6-OHDA was tracked by bromodeoxyuridine labeling. Two weeks after transplantation, in animals transplanted with NPCs, we found evidence of elevated host subventricular zone NPC proliferation, neurogenesis, and migration to the graft site. In these animals, we also observed a significant preservation of striatal tyrosine hydroxylase (TH) expression and substantia nigra TH cell number. We have seen no evidence that neuroprotection is a product of DA neuron replacement by NPC-derived cells. Rather, the NPCs expressed glial cell line-derived neurotrophic factor (GDNF), sonic hedgehog (Shh) and stromal cell derived factor 1 alpha (SDF1 α) providing a molecular basis for the observed neuroprotection and endogenous NPC response to transplantation. In summary, our data suggests plausible synergy between exogenous and endogenous NPC actions, and that NPC implantation before the 6-OHDA insult can create a host microenvironment conducive to stimulation of endogenous NPCs, and protection of mature nigral neurons.

Keywords

neuroprotection; neural stem cells; neurogenesis; transplantation; Parkinson's disease; 6-hydroxydopamine; growth factor

INTRODUCTION

Stimulation of *endogenous* (host) neural precursor cells (NPCs) and *exogenous* NPC transplantation provide alternate promising options for Parkinson's disease (PD) therapy. NPCs show the ability to integrate into the host, differentiate into DA neurons, be neuroprotective, and in several cases lead to some functional recovery after transplantation (Armstrong et al., 2002; Daadi and Weiss, 1999; Madhavan et al., 2007; Ourednik et al., 2002; Rafuse et al., 2005; Richardson et al., 2005; Shim et al., 2007; Studer et al., 2000; Studer et al., 1998; Svendsen et al., 1997; Wagner et al., 1999; Yan et al., 2001; Yasuhara et al., 2006). These results suggest the possibility of using NPC transplants for treating PD.

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However their poor survival, slow maturation, and low and unstable differentiation rate to dopamine neurons currently prevents any meaningful clinical applications. More recently, efforts have been extended to stimulating and recruiting endogenous precursors residing within the adult CNS in PD. In this regard, there is convincing evidence in PD models (6-OHDA) that growth factor treatment activates endogenous neurogenesis and migration of precursor cells from the subventricular zone (SVZ), an area known to support persistent generation of neural stem cells even in adult brain (Cooper and Isacson, 2004; Fallon et al., 2000; Mohapel et al., 2005). Also, there is evidence that neurogenesis may be occurring (Zhao et al., 2003) or induced to occur (Van Kampen and Eckman, 2006), in the normal and PD affected brain. While activation of endogenous NPCs appears to occur, the response fails as a source of meaningful compensation. Hence, techniques to attract optimal numbers of cells, differentiate them into relevant types of neurons and/or glia, form appropriate cell-contacts and execute the various functions of that brain region need to be developed. Therefore, in summary many challenges still need to be overcome before the strategy of recruiting endogenous stem cells or transplanting NPCs can be utilized to attain any realistic PD therapy.

As indicated above, so far, therapeutic strategies in PD precursor cell research have been divided into studying cell transplantation or endogenous cell stimulation, separately. Since future cell therapies for PD will likely involve a combination of both stimulation of endogenous cells and transplantation, it is essential to study these two modes of precursor cell therapy jointly and determine whether and how communication between these two cell types contributes to therapeutic effects. Also, it is known that the destiny of a stem/precursor cell depends critically on its 'niche' or local environment (Jiao and Chen, 2008). In the case of transplanted NPCs, since endogenous NPCs are also present in their environment, interactions between these two cell types may play a critical role in determining the behavior and fate of both, and ultimately the impact on neural protection and repair. Therefore, it is important to investigate the interactions between exogenously transplanted and endogenously present NPCs since it will provide important information regarding significant aspects of the niche and contribute towards development of realistic cell therapies for PD.

The overall goal of our studies is to examine the role of and mechanisms underlying an endogenous neural precursor cell response in transplant induced therapeutic effects. The present study was undertaken as a first step to determine whether NPCs when transplanted prior to the toxic insult could stimulate endogenous NPCs and induce neuroprotection in a parkinsonian rat model, and in the process characterize the extent of neuroprotection, the extent of graft-induced endogenous precursor response, the distribution of graft and host progenitors, and initial identification of factors that may mediate stimulation of endogenous precursors and neuroprotection. We hypothesized that there exists a synergism between the actions of endogenous and grafted neural precursor cells after transplantation which would lead to neuroprotection in a 6-OHDA rat model of Parkinson's disease. In our study, human placental alkaline phosphatase (hPAP) (Han et al., 2002; Mujtaba et al., 2002) labeled NPCs isolated from the subventricular zone (SVZ) of newborn transgenic rat pups were transplanted unilaterally into the striatum and substantia nigra of host rats enabling us to follow the fate of these cells after transplantation. Subsequently, 6-OHDA was administered ipsilaterally into the median forebrain bundle to induce degeneration of the nigrostriatal system, and the reaction of the endogenous NPCs to the transplantation and 6-OHDA lesioning was tracked by bromodeoxyuridine (brdU) labeling. Our studies, for the first time, focus on interrelationships between endogenous (resident) and exogenous (transplanted) NPCs and provide novel information on how a combined action of these two cell types can lead to neural protection in a PD model.

Materials and Methods

In Vitro

NPC culture—NPCs isolated from SVZs of newborn transgenic Fischer 344 rats expressing hPAP were grown under standard conditions in uncoated dishes and serum-free neurobasal-A medium (*Invitrogen, Carlsbad, CA*) supplemented with 2% B27 (*Invitrogen, Carlsbad, CA*), 20 ng/ml epidermal growth factor (EGF, *Cell Sciences, Canton, MA*), 10 ng/ml basic fibroblast growth factor (bFGF, *Cell Sciences, Canton, MA*), 5 ug/ml heparin (*Stemcell Technologies, Vancouver, BC, Canada*), 500 μ m glutamax, 200 units/ml penicillin, and 200 μ g/ml streptomycin (*Invitrogen, Carlsbad, CA*), at 37°C.

Immunocytochemistry and cell counts—Cells were grown on polylysine/laminin (0.1 mg per ml/5 μ g per ml) coated coverslips, fixed with 4% paraformaldehyde (PFA), rinsed with phosphate buffered saline (PBS, pH-7.4) and blocked. Primary antibodies were diluted in blocking solution (5% goat serum, 0.2% bovine serum albumin (BSA), 0.1% triton X-100 in PBS) and preparations incubated overnight at 4°C. Specific binding was revealed with the appropriate secondary antibodies (1:500) conjugated to Alexa Fluor 488, 594 or 647 (*Invitrogen-Probes, Carlsbad, CA*). Primary antibody dilutions were as follows: Nestin (1:500), Tuj1 (1:500), RIP (1:1000), TH (1:4000), GAD (1:500) from (*Millipore-Chemicon, Danvers, MA*); hPAP (1:1000, *Accurate, Westbury, NY*); S100 β (1:1000, *Sigma, St Louis, MO*). Detailed information on all antibodies is available in the antibody characterization subdivision within the methods section. Primary antibody replaced with matched serum or non-immune isotype specific controls and secondary antibody deleted controls were performed. Quantification of hPAP cells double labeled with tuj1, S100 β , RIP, TH or GAD were conducted through a 40X objective using a Zeiss Axioplan 2 microscope. 15–20 visual fields, from 3–4 separate sets of cultures were counted. Each separate culture set contained at least 3 parallel cultures.

In vivo

Animals and Surgery—Lesions and grafts were performed in male Fischer344 rats (250–275g, Harlan, Indianapolis, IN) which were housed at the laboratory animal medical services (LAMS) facility at the University of Cincinnati and kept on a 12-hour light-dark cycle. All applicable laws, regulations, and guidelines from the National Institutes of Health for animal use and care were followed. The Institutional Animal Care and Use Committee (IACUC) at the University of Cincinnati approved this study. *NPC transplants*: Infusion of undifferentiated hPAP expressing NPC's (50,000 cells/ μ L, 2 μ L/site) in the striatum (AP +0.2, ML +3, DV -5.5 from bregma) and substantia nigra (AP -5.3, ML +2.5, DV -7 from bregma) was conducted under stereotaxic guidance in a single session. NPC transplantation from hPAP rats does not require immune suppression as these rats are raised on the same Fischer344 background as the host rats. *6-OHDA lesions*: 1 μ l of 6-OHDA (4 μ g/ μ l) was infused into the MFB (AP: -4, ML: +1.2, DV:-7.5) at 1 week after NPC transplantation.

At the prescribed time points after NPC grafting, animals were sacrificed via barbiturate overdose, perfused with 4% PFA, removed brains post-fixed in 4% PFA solution, and sectioned (40 μ m) on a freezing sliding microtome for morphological studies.

BrdU administration—To label proliferating endogenous NPCs, rats were injected i.p. with brdU (50 mg/kg/12 hrs) for 3 days before transplantation (*brdU1*) or for 2 weeks after transplantation (*brdU2*) [see experimental timeline below]. Application of brdU (a) before transplantation labels pre-existing naive resident NPCs present in the germinal niches (SVZ and dentate gyrus of the hippocampus) which later respond to ensuing NPC transplantation and 6-OHDA application, and (b) after transplantation labels the immediate response of

endogenous NPCs in germinal niches and other cells in the parenchyma following transplantation and 6-OHDA application.

Experimental paradigm—The experimental timeline is depicted below.

The experimental groups were as follows: Sham transplant and sham lesion (N=6); Sham transplant and 6-OHDA lesion (N=6); Dead ventral mesencephalic cells and 6-OHDA (N=5); NPC transplant and 6-OHDA lesion (N=13); NPC transplant and sham lesion (N=6); Intact (8).

Immunohistochemistry—Sections were blocked [(10% goat serum, 0.5% triton-X in tris buffered saline (TBS, pH-7.4)] and incubated with the primary antibody overnight at room temperature (RT) or at 4°C for 48 hrs. Primary Abs were detected in a 2-hr-incubation at RT with secondary antibodies (1:300) coupled to the fluorochromes Alexa Fluor 488, 555, 594, or 647 (*Invitrogen-probes, Carlsbad, CA*) for immunofluorescence. Alternatively, 3,3' diaminobenzidine (DAB)- or vector blue-staining with biotinylated secondaries and ABC peroxidase kit (*Vector labs, Burlingame, CA*) was performed. Primary Abs used were as follows: BrdU (1:200), Nestin (1:200), RIP (1:500), TH (1:4000) all from *Millipore-Chemicon, Danvers, MA*; SDF-1 α (1:250) from *Abcam, MA*; Shh (1:200), GDNF (1:500), Dcx (1:400) from *Santacruz, CA*; hPAP 1:1000 from *Accurate, Westbury, NY*; S100 β 1:500 from *Sigma, St Louis, MO*. Detailed information on all antibodies is available in the antibody characterization subdivision within the methods section. Primary antibody replaced with matched serum or non-immune IgG1 isotype specific controls and secondary antibody deleted controls were performed.

Antibody characterization—The immunogen for the anti-nestin mouse monoclonal antibody (clone rat-401, Millipore-Chemicon, #MAB353, lot #0703053691) was nestin purified from rat embryonic spinal cord. As indicated by the supplier, rodent nestin protein specificity has been confirmed in rat and mouse (does not react with human) and it is recognized as a 200–220 kDa band in reducing gels of newborn rat or mouse cell extracts. This antibody has been widely used (eg. Okada et al., 2006) and stains neural stem cells of the ventricular zone of the rat brain by immunofluorescence, as indicated on the company product page. In our study, nestin stained only neural stem cells but not their differentiated progeny and these cells showed expected morphology and distribution.

The rabbit monoclonal anti-Tuj1 antibody (clone Tuj1 1–15–79, Covance #MRB435P, lot #14922901) was raised against microtubules derived from rat brain. As indicated on the manufacturer sheet, it is well characterized and highly reactive to neuron specific class III β -tubulin and does not react with β -tubulin found in glial cells. In western blots using rat brain lysates it is recognized as a 50 kDa band. On immunohistochemical analyses in our study we observed an expected staining pattern in vitro and in vivo as indicated on the company product page and by Amoh et al., 2005.

The mouse monoclonal anti-S100 β antibody (clone SH-B1, Sigma # S2532, lot #085K4880) was derived from SH-B1 hybridoma produced by the fusion of mouse myeloma cells and splenocytes from an immunized mouse. As indicated on the manufacturer's product sheet, purified bovine S100b preparation was used as an immunogen. This antibody recognizes an epitope located on the β chain but not on the α -chain of S-100, and does not react with other members of the EF-hand family such as calmodulin, parvalbumin, intestinal calcium binding protein and myosin light chain. The antibody has been extensively characterized by immunohistochemistry, western blotting (20 kDa band in mouse homogenates as shown by Liao et al., 2008), ELISA and dot blot as indicated on the company product sheet. In our study, the S100 β antibody stained cells with the classic morphology and distribution of astrocytes as shown previously by Seki et al., 2007.

The immunogen for the mouse anti-oligodendrocytes (RIP) monoclonal antibody (clone NS-1, Millipore-Chemicon #MAB1580, lot #LV1486518) was rat olfactory bulb extract. As indicated on the company product page it specifically stains oligodendrocytes and their myelin sheaths in both early and mature oligodendrocytes. The RIP antibody has been well utilized to stain oligodendrocytes as indicated on the product page (eg. Mitsui et al., 2005). In our study we observed typical oligodendrocyte morphology in the differentiated progeny of neural stem cells stained by the RIP antibody.

The anti-human placental alkaline phosphatase (hPAP) rabbit polyclonal antibody (Accurate #YSRTAHP537, lot #191107) was raised against purified human placental alkaline phosphatase. The antibody shows expected staining pattern as previously shown by Lepore and Fischer, 2005. In our study, the antibody specifically stained only the grafted precursor cells which had been isolated from hPAP expressing transgenic animals with no staining observed elsewhere in the rat brain or in non-grafted animals.

The mouse monoclonal anti-tyrosine hydroxylase (TH) antibody (clone LNC1, Millipore-Chemicon #MAB318, lot #0509010596) was raised against purified TH from PC12 cells. This antibody which has been widely used (eg. Sanchez-Gonzalez et al., 2005) recognizes an epitope on the outside of the regulatory N-terminus and detects a 59–61 kDa band on western blotting using human and rat brain tissue. It does not cross-react with dopamine- β -hydroxylase, phenylalanine hydroxylase, tryptophan hydroxylase, dihydropteridine reductase, sepiapterin reductase, or phenethanolamine-*N*-methyl transferase. It is shown to stain embryonic rat mesencephalic dopaminergic neurons and rat dorsal root ganglia as depicted on the company product page and stained cells with the typical morphology and distribution of TH cells in our study.

The immunogen for the mouse monoclonal anti-glutamic acid decarboxylase (GAD) antibody (Millipore-Chemicon #MAB351, lot # 051001366) was purified rat brain GAD and recognizes the lower molecular weight (65 kDa) GAD isoform on rat brain homogenates. When used in our study it showed expected pattern of staining as shown by Park et al., 2004.

Mouse monoclonal anti-bromodeoxyuridine (brdU) antibody (Millipore-Chemicon #MAB3222, lot # LV1378389) reacts specifically with bromodeoxyuridine and also recognizes brdU when incorporated into nuclear DNA. The immunogen for this antibody was a fusion of mouse myeloma cells with spleen cells from a mouse intraperitoneally injected with brdU conjugated to BSA. In our study, brdU antibody stained cells in the subventricular zone and hippocampus in an expected morphological and distribution pattern (Kuhn et al., 1996). No staining was seen without a prior hydrochloric acid (HCl) hydrolysis step or when the antibody was used to stain tissue sections from rats that had not been injected with brdU.

The anti-doublecortin goat polyclonal antibody (C-18, Santacruz biotechnology #sc8066, lot #D0808) was raised against a peptide mapping (AA sequence: 385–402, PMID: 18231966) at the C-terminus of doublecortin of human origin. It is specific for doublecortin of mouse, rat, and human origin by immunoprecipitation, western blotting and immunohistochemistry and does not crossreact with a related protein KIAA0369. It recognizes a 45 kDa protein on western blotting using SK-N-SH whole cell lysates and mouse embryo extract as shown on the company product page. Also, it stains newly formed neurons which show expected morphological staining pattern and distribution as reported by others before (eg. Gleeson et al., 1999) and on the manufacturer sheet.

The anti-glial cell-line derived neurotrophic factor goat polyclonal antibody (D-20, Santacruz biotechnology, #sc328, lot #H1307) was raised against a peptide mapping near the C-terminus (AA sequence: 186–205, PMID:15618882) of GDNF of human origin. It detects a 15 kDa band on western blotting on mouse and rat brain extracts and in our study stained cells with the typical expected cytoplasmic staining pattern as shown on the company product page.

The goat polyclonal anti-sonic hedgehog (Shh) antibody (N-19, Santacruz #sc1194, lot #G1807) was raised against a peptide mapping at the N-terminus (AA sequence: 30–48, PMID: 18465790) of mature Shh of human origin. Using the human neuroblastoma cell line IMR-32 whole cell lysate, it detects a 45 kDa band on western blots and stains cells with a staining pattern expected of Shh expressing cells as shown on the product sheet.

The anti-stromal cell derived factor 1 alpha (SDF1 α) rabbit polyclonal antibody (Abcam #ab25117, lot #366049) was generated by using E-coli expressed mouse SDF1 α as the immunogen. It detects a 11 kDa band on western blots of mouse pancreas, thymus and brain and in our study showed expected staining pattern on immunohistochemical analyses as shown by Lima e Silva et al., 2007.

Stereology and Dcx cell counts

Stereology: Stereological probes were applied using a BX52 Olympus microscope (Olympus America Inc.) equipped with Microbrightfield stereological software and a Microfire CCD camera (Optronics, Goleta, CA) by the optical fractionator method. Briefly, once section thickness was determined, guard zones were set (2 μ m at the very top and very bottom of the section that are not included in the counting area) which prevent double counting of cells. Cells were counted under the 60X oil immersion objective by lowering the counting frame through the tissue at 1–2 μ m intervals and marking each cell that is counted. The substantia nigra and SVZ contours were drawn at low magnification (1.25x). TH cells were counted in sections 480 μ m apart using a grid size of 170 $\times$ 100 μ m and counting frame size of 50 $\times$ 50 μ m. For the brdU counts, counts were conducted in the dorsolateral SVZ in sections at 480 μ m intervals between the genu of the corpus callosum and anterior commissure crossing as outlined by the Gotts and Chesselet study (Gotts and Chesselet, 2005). The grid size used was 100 $\times$ 100 μ m and the counting frame was 75 $\times$ 75 μ m. The Gundersen method for calculating the coefficient of error was used to estimate the accuracy of the optical fractionator results (Gundersen et al., 1999; West and Gundersen, 1990) coefficients were generally less than 0.1.

BrdU/Dcx cell counts: Cell counts for BrdU/Dcx double labeled cells in the striatum adjacent to the graft were obtained in sections at 480 μ m intervals under a 40X objective using epifluorescence. The double labeling was validated using a confocal laser-scanning microscope.

Microscopy and Image production—Non-fluorescently immunolabeled sections were analyzed on an OlympusBX60 microscope and images captured with a NikonDXM1200 digital camera. Fluorescently immunolabeled sections were analyzed on a Zeiss Axioplan 2 microscope and images were captured with an Orca-ER cooled, B&W CCD camera coupled to the microscope or a Zeiss LSM510 confocal laser scanning microscope. Confocal imaging and Z sectioning was performed at 1µm intervals in order to verify colocalization of hPAP or brdU with other markers. Images were acquired and reconstructed using the Zeiss LSM software. Figures were produced in Photoshop 7.0, by adjusting saturation, sharpness and brightness only as necessary to best approximate the immunostaining as viewed directly under the microscope.

Statistical analyses—Graphpad prism 5 software was used for statistical analyses. For comparisons between two or more groups, ANOVA followed by Dunnett's post hoc test for multiple comparisons with the control, or Bonferoni's test for multiple comparisons between treatment groups was conducted. For comparisons between 2 groups a Student's t-test was performed. A Pearson correlation analyses was performed between substantia nigra TH cell counts and SVZ brdU cell counts. Differences were accepted as significant at $p < 0.05$.

RESULTS

The aim of our study was to determine whether transplantation of donor NPCs prior to a 6-OHDA insult would induce proliferation and migration of host endogenous precursors and result in neuroprotection in hemi-parkinsonian rats.

***In vitro* and *in vivo* characterization of human placental alkaline phosphatase expressing subventricular zone neural precursors**

We utilized neural precursor cells (NPCs) derived from the subventricular zone (SVZ) of newborn transgenic F344 rat pups expressing human placental alkaline phosphatase (hPAP) (Han et al., 2002; Mujtaba et al., 2002) in order to distinguish grafted NPCs from host cells with immunohistochemistry after transplantation. We first characterized the *in vitro* nature of the hPAP expressing NPCs. As depicted in figure 1, the undifferentiated SVZ-NPCs expressed hPAP (Fig. 1a, red) and the immature neural cell marker nestin (Fig. 1b, green). Upon differentiation following mitogen withdrawal cells began to express neuronal (tuj1, Fig. 1c, red), astrocyte (S100β, 1d, red) and oligodendrocyte (RIP, Fig. 1e, red) markers, confirming their multipotentiality and identity as neural precursor cells. Amongst the neuronal population, many were GAD expressing (Fig. 1f, red) while a small proportion expressed TH (Fig 1g, green). The percentage of hPAP neural precursor cells differentiating into neurons, glia and the two neuronal subtypes are depicted in figure 1h. Subsequently, these hPAP expressing NPCs were transplanted into host Fischer 344 rats according to the paradigm shown in the experimental timeline (please see materials and methods, page 7). Figure 2 demonstrates that the NPCs continued to show robust expression of hPAP immediately prior to (Fig 2a, b) and 2 weeks after transplantation (2c, d) allowing us to track the fate of the transplanted NPCs.

Neural precursor cell transplantation prior to 6-OHDA lesion induces protection of the nigrostriatal system

As a next step, we analyzed the effect of NPC transplantation on the damaged host nigrostriatal system. We observed that there was significant preservation of host striatal tyrosine hydroxylase (TH) expression in the striatum immediately surrounding grafted NPCs (Fig 3a, b, * indicates graft site) in 6-OHDA lesioned animals that had received prior NPC grafts as compared to the ones that did not. Also, in these animals, there was significant preservation of the TH cell number in the substantia nigra (SN) as indicated in figure 3c–f.

Stereological cell counts through SN confirmed these results and indicated that NPC transplantation had significantly ($p < 0.01$) prevented the reduction in TH-positive cell number after 6-OHDA application in the transplanted animals as compared to the animals that received 6-OHDA with no grafts (Fig 3g, $76.5 \pm 3.46\%$ in NPC grafted animals vs $41.18 \pm 3.52\%$ in sham grafted).

Transplantation of neural precursor cells induces endogenous precursor cell proliferation, migration to the graft site and neurogenesis

After confirming the presence of neuroprotection in our model, we then assessed the response of endogenous NPCs to transplantation and 6-OHDA application by means of bromodeoxyuridine (brdU) labeling. At two weeks after transplantation we observed an increased intensity of brdU staining in the SVZ of lesioned and grafted animals (brdU1 & 2, Fig 4c, d) as compared to the non-grafted animals receiving 6-OHDA (Fig 4b) or vehicle (Fig 4a). Also, dead cell grafted animals receiving 6-OHDA did not show any increase in brdU labeling. Stereological counts through the SVZ indicated that there was a significantly greater number of brdU labeled endogenous NPCs in lesion + graft animals (Fig 4i, 156.93 ± 8.52 in NPC transplanted vs 100.19 ± 4.2 in sham transplanted, $p < 0.001$). We also studied the expression of nestin, an immature NPC marker, in the SVZ and observed that there was greater nestin immunoreactivity in the SVZ of grafted animals as compared to the sham transplanted ones (Fig 4e–h). We did not observe any increased brdU or nestin labeling in the SVZ of NPC grafted animals which did not receive subsequent 6-OHDA infusion. These results corroborated the presence of elevated SVZ brdU expression and cell numbers, and indicated that NPC transplantation along with 6-OHDA application had stimulated proliferation of endogenous SVZ precursors. Further, we analyzed the relationship between the magnitude of protection of TH neurons and magnitude of increase in brdU cells in the SVZ. Interestingly, we found that there was a strong correlation between the endogenous NPC response in the SVZ (brdU cell numbers) and neuroprotection of dopaminergic neurons (TH-positive cell number) in the substantia nigra ($r = 0.70$, $p < 0.03$) as depicted in the graph in figure 4j suggesting a plausible endogenous NPC role in the observed nigrostriatal neuroprotection.

In terms of the effect of NPC transplantation on the migration of the endogenous precursors, we observed many host endogenous NPCs (brdU-positive, hPAP-negative cells) surrounding the graft in both the striatum and the nigra in NPC transplanted rats two weeks after transplantation (arrow in Fig 5a). Particularly, we found the presence of such brdU-positive host cells adjacent to the graft in rats treated with brdU before transplantation (brdU1 paradigm in materials and methods on page 7, arrows in figure 5b point to brdU expressing cells). Figure 5c depicts a confocal z section through the grafted region in an NPC transplanted animal which received brdU prior to transplantation, and shows a clear presence of endogenous brdU cells (blue nuclei), their distribution and their close relationship with the exogenous hPAP (green) cells. These data together suggested that endogenous SVZ precursors that had divided prior to grafting or lesion had migrated to the graft site.

After confirming the presence of increased endogenous precursor cell proliferation and migration in the grafted toxin treated animals, we then investigated whether this endogenous NPC response to transplantation was also accompanied by increased neurogenesis in these animals. We immunostained for doublecortin (dcx), a marker for migrating neuroblasts, and found an increased expression of dcx in the SVZ of lesioned rats which had received NPC-transplants (Fig 6b) compared to lesioned sham-grafted controls (Fig 6a). In addition we observed dcx cells in the region proximal to the graft (arrows in 6c). These dcx labeled cells were brdU positive but hPAP negative as indicated by the confocal images in figure 6e–h, indicating that they were of host origin. Counts in the striatum of these dcx cells in the area

surrounding the graft confirmed that they were present in significantly greater numbers in the grafted animals as compared to the sham controls ($p < 0.01$, Fig 6d). These results indicated that NPC transplantation had induced neurogenesis in the SVZ, and summoned endogenous NPCs to the graft site. We did not find any endogenous NPC differentiation into TH-positive neurons.

Phenotypic characterization of transplanted neural precursor cells

In terms of the distribution and differentiation of the grafted cells we observed the following: Most of the grafted cells at 2 weeks post-transplantation remained at the site of transplantation (Figure 7a and Figure 2c, d). Examination of phenotypic markers of grafted cells indicated that although some of the grafted hPAP labeled NPCs still expressed the immature NPC marker nestin (Fig 7a, hPAP/nestin double-positivity seen as pink), a large proportion (~50%) of the hPAP labeled grafted cells transformed into S100 β positive astrocytes (Fig 7b, hPAP/S100 β double positive cells seen in cyan). A smaller proportion expressed the early neuronal marker Tuj1 (~20%, Fig 7c, hPAP/tuj1 double positive cells seen in cyan), with extremely rare expression of TH (<<1%).

Transplanted and endogenous neural precursors express specific chemokines and growth factors

Our data so far indicated that NPC transplantation before the 6-OHDA insult stimulates an endogenous precursor cell response (specifically proliferation, migration and neurogenesis) and neuroprotection. Also, there was virtually no differentiation of grafted NPCs into TH neurons indicating that alternate mechanisms besides cell replacement had led to neuroprotection. Therefore, we initiated a preliminary investigation of plausible molecules that could lead to the abovementioned phenomenon. We looked for expression of selected growth factors and chemokines by the grafted NPCs which could stimulate endogenous NPCs and contribute to the neuroprotection. Specifically, we immunostained for brain-derived neurotrophic factor (BDNF), GDNF, Shh, neurotrophin 3 (NT-3) and SDF1 α , which have been implicated in endogenous NPC stimulation and/or are relevant to neuroprotection of the nigrostriatal DA system (Angot et al., 2008; Baquet et al., 2005; Benraiss et al., 2001; Ericson et al., 2005; Espejo et al., 2000; Imitola et al., 2004b; Jiang et al., 2006; Kobayashi et al., 2006; Miao et al., 1997; Shyu et al., 2008). From amongst these factors, we observed significant expression of GDNF (8Aa-c), Shh (8Ad-f) and SDF1 α (8Ag-i) by the grafted NPCs in both the striatum and the substantia nigra. We conducted confocal microscopy to look for colocalization and confirm the expression of these factors by the grafted cells as depicted in Figure 8B (a-i). Interestingly, in addition to the grafted NPCs, some of the host NPCs (brdU positive but hPAP negative cells) present in the area surrounding the graft also expressed Shh but only in the animals that had received NPC grafts before the 6-OHDA insult suggesting some reciprocal signaling between these two NPC populations. Confocal images in figure 8Bj, k, l, m are from a NPC transplanted and lesioned animal that had received brdU prior to grafting showing endogenous NPC expression of Shh.

DISCUSSION

Our study indicates that neural precursor cell (NPC) implantation prior to a toxic insult can stimulate significant endogenous NPC proliferation, migration and neuronal differentiation associated with nigrostriatal protection in a rat model of Parkinson's disease. The data also suggests that graft-expressed GDNF, SHH and SDF1 α may play a role in initiating the endogenous NPC response and lead to neuroprotection in our model. The study provides novel information on endogenous NPC responses associated with neuroprotection in a rat PD model, and provides targets that can be manipulated to augment the endogenous NPC

population available to respond to signals from the grafted NPCs and lead to optimal cell therapies.

It is known that the destiny of a stem/precursor cell depends critically on its 'niche' or local environment (Jiao and Chen, 2008). In the case of transplanted NPCs, since endogenous NPCs are also present in their environment, interactions between these two cell types may play a critical role in determining the behavior and fate of both, and ultimately the impact on therapeutic outcome. In this context, recent reports indicate that transplantation of other types of precursor cells such as human bone marrow cells, marrow stromal cells and CD34⁺ cells can stimulate endogenous NPCs and induce SVZ and hippocampal neurogenesis (Mahmood et al., 2004; Munoz et al., 2005; Taguchi et al., 2004). In terms of NPCs and PD models, a study by Yasuhara and colleagues (Yasuhara et al., 2006) recently reported stimulation of endogenous neurogenesis upon transplantation of immortalized HB1F3 cells using a same day lesion-transplant paradigm. Although the study did assess the presence and magnitude of endogenous neurogenesis, their focus was on whether transplantation of human NSCs cloned by *v-myc* gene transfer (HB1F3 cells), is a feasible therapeutic option for Parkinson's disease. The study reported about a 140% increase in endogenous neurogenesis as assessed via brdU/dcx double-positive cell counts in the SVZ in response to transplantation of the HB1F3 cells, with no migration, differentiation into TH-positive neurons or indication of participation of the endogenous NPCs in the neuroprotection that they observe. The authors suggest a possible connection between unknown graft-derived secreted factors and the enhanced neurogenesis, and comment that the unknown factors probably possessed neurogenic but not migratory triggers. Our studies support the Yasuhara et al report and the concept that it is important to investigate endogenous neurogenesis as it is an essential factor which may contribute to transplanted NPC-induced neuroprotection. The novelty of our study lies in its focus on the endogenous response to transplantation, the transplantation of primary neural precursor cells *prior* to the 6-OHDA insult resulting in neuroprotection, observation of a significant endogenous NPC response (cell proliferation, migration and neurogenesis) in response to transplantation, and the presence of a strong correlation between the endogenous SVZ response and substantia nigra TH cell neuroprotection. In addition, our study reports a robust expression of three factors, GDNF, SHH and SDF1 α which have the ability to induce neurogenesis and neuroprotection providing us targets for future manipulation that may help enhance the endogenous NPC responses and neuroprotection. Studies spanning a longer period, and which include behavioral analyses to further assess neuroprotection and endogenous NPC stimulation will be conducted in the future.

The abovementioned phenomenon of so called "transplantation-induced neurogenesis" may be explained by grafted NPCs acting as biological minipumps for release of growth factors and chemokines that stimulate plastic responses from the host, including the stimulation of endogenous neurogenesis. In support of this hypothesis NPCs previously have been shown to spontaneously produce a variety of growth and plasticity promoting factors including GDNF, neurotrophin-3 (NT-3), brain derived neurotrophic factor (BDNF), and nerve growth factor (NGF) (Imitola et al., 2004a; Lu et al., 2003; Ourednik et al., 2002; Ryu et al., 2004; Teng et al., 2002; Yasuhara et al., 2006). Also, recent studies by Mohapel et al (2005) and Cooper and Isaacson (2004) provide compelling evidence demonstrating that treatment with growth factors activates endogenous neurogenesis in the SVZ of PD model rats. Our experiments support the above studies in that we also observed that the grafted NPCs expressed particular growth factors and chemokines namely GDNF, Shh and SDF1 α which show the ability to stimulate endogenous NPCs and also hold particular relevance to the nigrostriatal system (Angot et al., 2008; Ericson et al., 2005; Imitola et al., 2004b; Kobayashi et al., 2006; Miao et al., 1997; Palma et al., 2005; Shyu et al., 2008; Tran et al., 2007). Additionally we found a significant increase in levels of endogenous precursor cell

proliferation in lesioned animals that had received prior NPC transplants. Interestingly, the observed levels of endogenous NPC proliferation assessed via brdU labeling were higher at a 156% increase at two weeks after SVZ-NPC transplantation compared with a 128% increase in SVZ cells expressing nestin after 4 weeks of TGF α administration in the Cooper and Isacson study (Cooper and Isacson, 2004). This result implies that transplantation of NPCs in the optimal time interval and anatomical sites can recruit the support of endogenous NPCs, and that a combined action of both transplanted and endogenous NPCs can be quite potent and a more advantageous route to utilize than growth factor therapy alone. Interestingly, we did not observe any increased cell proliferation in the SVZ of NPC grafted animals which did not receive subsequent 6-OHDA infusion, indicating that both the graft and the lesion are necessary to see an endogenous NPC response. It remains possible though that there had been a short-lived endogenous NPC response after transplantation. Future studies shall follow the course of the endogenous NPC response over time after transplantation and 6-OHDA application.

In our study, it is conceivable that the transplanted NPCs via expression of GDNF, Shh and SDF1 α acted directly or indirectly via mature host CNS cells (e.g. astrocytes and microglia), to stimulate endogenous NPCs to proliferate, migrate, and differentiate into neurons. The issue of how far such trophic factors can diffuse to have direct effects on pertinent cells is still not clear, but recent studies indicate that GDNF and Shh can spread considerable distances in the striatum to stimulate cells in the SVZ (Charytoniuk et al., 2002; Kobayashi et al., 2006), and SDF1 α has been shown to mediate long-distance NPC migration towards injury sites where it has been expressed (Charytoniuk et al., 2002; Imitola et al., 2004b; Robin et al., 2006). With regards to the specific actions of these expressed factors, GDNF has been shown to have neuroprotective and trophic actions on dopaminergic neurons when injected into the striatum or substantia nigra (Dowd et al., 2005; Ericson et al., 2005). Also GDNF has been shown to promote SVZ neurogenesis and migration of newly born neuroblasts into the striatum (Kobayashi et al., 2006). Shh has been shown to be a critical factor for the survival and differentiation of dopaminergic neurons during development and in protecting them against toxic insults in the adult brain (Hynes et al., 1995; Miao et al., 1997; Tsuboi and Shults, 2002). In addition, Shh displays a chemoattractive activity in vitro on SVZ-derived neuronal progenitors and is also known to control stem cell behavior in the postnatal and adult brain (Angot et al., 2008; Palma et al., 2005). In terms of the chemokine SDF-1 α , it has been shown to be able to direct migration of NPCs to sites of injury (Imitola et al., 2004b; Robin et al., 2006) and also play neuroprotective roles (Shyu et al., 2008). Thus one or all of these factors and others may be playing a critical role in the observed endogenous NPC response to transplantation and neuroprotection. Future studies shall determine the role and relative participation of these factors in observed neuroprotection and endogenous NPC response to neural precursor transplantation, and also particular graft and host cell types expressing these factors.

The current study certainly does not imply that growth factor and chemokine expression solely underlie the observed neuroprotection. Other factors like activation of toll-like receptors (TLRs) may have contributed to the observed neuroprotection and NPC interactions and need to be investigated (Martino and Pluchino, 2007). Specifically, TLRs have been shown to modulate NPC proliferation and neurogenesis and also contribute to neuroprotection. A study by (Rolls et al., 2007) suggests a role for TLR2 in neurogenesis and TLR3 has been shown to trigger the production of neuroprotective mediators by (Bsibsi et al., 2006).

A novel and important finding in our studies is that they point to the likelihood of synergistic interactions between endogenous and exogenous NPCs after transplantation. First, correlation analyses indicated a strong relationship between the magnitude of

endogenous precursor cell proliferation and the magnitude of neuroprotection of substantia nigra TH neurons, indicating that the endogenous NPCs may make a significant contribution to the observed neuroprotection (Fig 4i). Second, endogenous NPCs proximal to the graft expressed Shh which also may have contributed to the observed neuroprotection. This result is supported by a previous study by Rafuse and colleagues (Rafuse et al., 2005) which showed that SVZ NPCs can express Shh and that Shh has neuroprotective properties. Such expression of Shh by the endogenous NPCs in combination with Shh from the grafted NPCs, might have contributed to significantly increasing the available Shh pool, and strengthened the neuroprotective response. Further it points to some possible reciprocal signaling between the two NPC populations (endogenous and exogenous) via Shh since SVZ NPCs have been shown to express the Shh receptor “patched”, and by extension could respond to SHH derived from grafted NPC (Angot et al., 2008). Finally, endogenous NPCs via their expression of Shh (which has shown to possess neuroprotective and NPC regulating abilities) might have supported the survival of the grafted NPCs in return and indirectly contributed to the resultant neuroprotection. Of course it is possible that transplanted cells and not the endogenous cells are mostly responsible for the observed neuroprotection. Future studies shall specifically address the role of endogenous precursors in the observed neuroprotection and the survival and fate of the transplanted NPCs, in order to confirm the existence of synergistic exogenous-endogenous NPC interactions in our model.

In summary, our study indicates that that exogenous NPC transplantation can provide cues for endogenous precursor cell stimulation and that both these NPC components collectively are associated with a host environment that supports neural repair and neuroprotection. Investigating the molecular basis of such communication between these two NPC types may help develop optimal cell therapies for PD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. In vitro characterization of SVZ neural precursor cells

Undifferentiated SVZ-NPCs expressed hPAP (Fig. 1a, red) and the immature neural cell marker nestin (Fig. 1b, green). Upon differentiation following mitogen withdrawal they began to express neuronal (tuj1, Fig. 1c, red), astrocyte (S100β, 1d, red) and oligodendrocyte (RIP, Fig. 1e, red) markers. In the neuronal population, many were GAD expressing cells (Fig. 1f, red) while a small proportion became TH cells (Fig 1g, green). The percent of cells differentiating into neurons, glia and the two neuronal subtypes two weeks post-differentiation are shown in figure 1h. Scale bars: 1a–f: 50μm, g: 15μm.

Figure 2. Human placental alkaline phosphatase (hPAP) expressing NPCs in vivo

Staining for hPAP allowed tracking of the fate of grafted NPCs after transplantation. Figure 2 shows that the NPCs were grafted in an undifferentiated state [expressed hPAP and the immature neural cell marker nestin] immediately prior to (2a, b) and 2 weeks after transplantation (2c, d). Scale bars: a, b-50 μ m, c, d -200 μ m. A magenta-green copy of the figure is available in the supplementary data section as figure 1.

Figure 3. NPC-mediated neuroprotection

Two weeks after neural precursor cell transplantation there was significant preservation of striatal TH expression (Fig 3a, Fig 3b) and substantia nigra TH cells (3c–f) in 6-OHDA lesioned animals that had received prior NPC grafts as compared to the ones that did not. Stereological cell counts through the central substantia nigra (SN) indicated that NPC transplantation had resulted in significant neuroprotection (Fig 3g). The lesioned but non-grafted animals showed about a 59% decrease in SN TH cell numbers whereas the NPC transplanted group showed only about a 24% decrease in comparison. ** $p < 0.01$, *** $p < 0.001$. Values are expressed as % of intact side and significance was determined using

ANOVA followed by Dunnetts or Bonferroni's multiple comparison test as appropriate.
Scale bars: 500 μ m.

Figure 4. Proliferation of brdU positive SVZ cells in response to grafted NPCs and 6-OHDA

At two weeks post-plantation we observed an increased intensity of brdU staining in the SVZ of lesioned-grafted animals (Fig 4c, Fig 4d) as compared to the non-grafted animals receiving 6-OHDA (4b) or vehicle (4a). Stereological counts through the dorsolateral SVZ indicated a marked increase in the number of brdU labeled proliferating precursors in lesioned-grafted animals in comparison to lesion-sham controls (significant at $p < 0.001$, figure 4i). Corroborating these results, we also found elevated SVZ nestin expression (indicating the presence of greater number of proliferating NPCs) in the grafted animals (4g,h) and considerably lower nestin immunoreactivity in non grafted but lesioned (4f) and sham (4e) animals. *** $p < 0.001$. Values are expressed as % of intact side and significance was determined using ANOVA followed by Dunnetts or Bonferroni's multiple comparison test as appropriate. Figure 4j illustrates the correlation analysis conducted between SN TH cell counts and SVZ brdU cell counts when both were both expressed as % of intact side. A significant ($r = 0.70$, $p = 0.03$) correlation was found between the increase in brdU and TH cell numbers suggesting a high probability of endogenous NPC role in the observed nigrostriatal neuroprotection. The correlation analysis was conducted using the Pearson's r calculation. Scale bars: a–f: 50 μ m

Figure 5. Migration of brdU positive SVZ cells in response to grafted NPCs and 6-OHDA

BrdU immunoreactive cells were found surrounding the graft in NPC-transplanted (arrow in 5a) animals two weeks after transplantation. The presence of brdU-positive cells in the rats treated with brdU before transplantation (*brdU1*, arrows in 5b) suggested that endogenous precursors probably from the SVZ had migrated to the graft site. The confocal image in 5c depicts a higher magnification of the endogenous brdU positive but hPAP negative neural precursors and their distribution in the region of the graft. Scale bars: a: 500 μ m, b: 100 μ m, c: 20 μ m.

Figure 6. NPC grafting induced SVZ neurogenesis and migration of doublecortin (dcx) positive neuroblasts

In grafted animals, we found an increased number of dcx expressing cells in the SVZ of NPC-transplanted animals (Fig 6b) compared to sham grafted controls (Fig 6a). We also observed dcx cells proximal to the graft (red, arrows in 6c) which were brdU positive but hPAP negative as confirmed by confocal microscopy (6e–h) indicating that they were of host origin. Counts in the striatum of dcx/brdU double-positive cells confirmed that they were significantly greater in number in the grafted animals as compared to control (Fig 6d). These data suggested that NPC transplantation had induced neurogenesis and migration of the endogenous NPCs to the graft site. * $p < 0.01$. Significance was determined using student's *t* test. Scale bars a–c: 50 μ m, e–h: 10 μ m. A magenta-green copy of the figure is available in the supplementary data section as figure 2.

Figure 7. In vivo differentiation of NPCs

Examination of phenotypic markers of hPAP expressing grafted cells indicated that while some of the grafted hPAP labeled NPCs still expressed the immature NPC marker nestin (7a, colabeled areas seen as pink). A large proportion expressed the glial marker S100 β (7b, colabeling seen as cyan). A smaller proportion expressed the early neuronal marker Tuj1 (7c, hPAP/tuj1 cyan colabeling. Scale bars in a–c: 200 μ m.

Figure 8. Expression of GDNF, SHH and SDF1 α by the grafted NPCs

We analyzed expression of selected growth factors and chemokines in order to begin to address some of the molecules that may underlie the observed neuroprotection and endogenous NPC response to transplantation. We observed significant expression of GDNF (8Aa–c), Shh (8A5d–f) and SDF1 α (8Ag–i) by the grafted NPCs. The colocalization of the three expressed factors with hPAP in the grafted cells was assessed and confirmed by confocal microscopy as depicted in figure 8Ba–i. Moreover brdU labeled endogenous neural precursors expressed Shh as shown in the confocal images in 8j–m, suggesting a plausible contribution of these cells to the inter-NPC signaling and neuroprotection. Scale bars in 8A:

200 μ m, 8Ba–m: 10 μ m. A magenta-green copy of the images is available in the supplementary data section as figure 3A and figure 3B.