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# In Vitro Generation and Transplantation of Precursor-Derived Human Dopamine Neurons

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The use of in vitro expanded human CNS precursors has the potential to overcome some of the ethical, logistic and technical problems of fetal tissue transplantation in Parkinson disease. Cultured rat mesencephalic precursors proliferate in response to bFGF and upon mitogen withdrawal, differentiate into functional dopamine neurons that alleviate motor symptoms in Parkinsonian rats (Studer et al. [1998] Nat. Neurosci. 1:290–295). The successful clinical application of CNS precursor technology in Parkinson disease will depend on the efficient in vitro generation of human dopaminergic neurons. We demonstrate that human dopamine neurons can be generated from both midbrain and cortical precursors. Transplantation of midbrain precursor-derived dopamine neurons into Parkinsonian rats resulted in grafts rich in tyrosine hydroxylase positive neurons 6 weeks after transplantation. No surviving tyrosine hydroxylase positive neurons could be detected when dopamine neurons derived from cortical precursors were grafted. Our data demonstrate in vitro derivation of human dopamine neurons from expanded CNS precursors and encourage further studies that systematically address in vivo function and clinical potential. J. Neurosci. Res. 65:284–288, 2001.


**Key words:** Parkinson disease; 6-hydroxydopamine; stem cells; neural transplantation; dopamine; human

Parkinson disease is a neurodegenerative disorder caused by a progressive loss of dopamine (DA) neurons in the midbrain. The focal nature of the degeneration makes the disease suitable for restorative therapy. In the last decade more than 300 Parkinsonian patients have received fetal transplants and there is good evidence of long-term survival and function of the grafts (Piccini et al., 1999). Because of limited availability of human fetal tissue and ethical controversy regarding its use, there has been an active search for alternative sources of DA neurons. DA cell lines or fetal cells derived from xenogeneic sources could serve as a substitute for human fetal tissue. Both types of cells, however, have significant disadvantages such as tissue safety (Isacson and Breakefield, 1998) and limited therapeutic effectiveness (Schumacher et al., 2000). DA neurons generated in vitro from a renewable pool of primary CNS precursor cells could provide a safe and effective alternative.

The current study addresses whether techniques developed for the in vitro generation of rat DA neurons (Studer et al., 1998) can be applied to human tissue. The term "CNS precursor cell" as opposed to "CNS stem cell" is used to reflect multiclonal origin of these cells. Our results demonstrate in vitro generation of DA neurons derived from human CNS precursors and in vivo survival of such cells in an animal model of Parkinson disease.

## METHODS AND MATERIALS

### In Vitro Studies

Brain tissue from a 7-week-old human fetus was obtained in accordance with NIH guidelines (access provided by Dr. E.O. Major). Ventral mesencephalic and cortical pieces were identified and staged using established anatomical landmarks (Evtouchenko et al., 1996), collected in Ca- and Mg-free HBSS and cut into 1 $mm^3$ tissue pieces. The tissue was mechanically triturated and plated at a density of $30 \times 10^3$ cells/$cm^2$ on culture plates precoated with polyornithine/fibronectin (Johe et al., 1996). Basic-FGF (20 ng/ml) was added daily and the medium consisting of DMEM/F12 with N2 supplement was changed every other day. Cell proliferation was assayed at Day 5 of proliferation by measuring BrdU incorporation (Boehringer-Mannheim Biochemicals, Indianapolis, IN, 10 μM for 24 hr) followed by immediate cell fixation and BrdU im-

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munohistochemistry (Studer et al., 1998). After 7 days of cell proliferation (90% confluence), cells were differentiated as monolayers by mitogen withdrawal and supplementation of the medium with 1 mM dibutyryl cyclic AMP (db cAMP) and 100 μM ascorbic acid (AA) (Sigma Chemical Company, St. Louis, MO) as described previously for rat midbrain precursors (Yan et al., 2001). Reverse-phase high performance liquid chromatography (rpHPLC) was carried out after 3 days of differentiation on the supernatant of cells incubated for 15 min either in HBSS (basal release) or in HBSS containing 56 mM KCl (evoked release). HPLC equipment and settings have been described previously (Studer et al., 1996, 1998).

### In Vivo Studies

All experimental procedures were done following NIH guidelines for animal care. Adult female Sprague–Dawley rats (150–200 g, Taconic farms) were housed under standard conditions with free access to food and water. Twelve μg of 6-hydroxydopamine (6-OHDA, Sigma) in 4 μL (0.01% AA in saline) were stereotactically injected (0.5 μL/min) in two sites along the right medial forebrain bundle (AP-4.4, lat −1.2 ventral −7.8 and AP −4.0, lat −0.8 ventral −8, (Paxinos and Watson, 2000). D-methamphetamine (5 mg/kg s.c, Sigma) induced rotations were quantified using an automated rotameter system (Macintosh Computer, NIH software Rotate 1.6) 2 and 3 weeks after 6OHDA lesion and 3 and 6 weeks post grafting. Animals with a mean rotation of > 6 clockwise turns (9.9 ± 0.9, n = 14) per min (60-min test period) were selected for transplantation. All animals were immunosuppressed with cyclosporine A (Neoral, Novartis, 10 mg/kg/day i.p.) starting 24 hr before grafting. This dose provides adequate immunosuppression as assessed by inversion of the CD4/CD8 ratio in FACS analyses (Sánchez-Pernaute, unpublished observations). Cultured cells (2 days of differentiation) were collected for grafting in HBSS containing 1% DNAseI (Worthington). Six μl of the resuspended cells (65–75,000 cells/μl were slowly (0.5 μL/min, 2 μL/mm) injected into the right striatum (AP + 1.0, ML −2.8 V −6 to %3 mm) using a 10 μl Hamilton syringe (animals receiving midbrain precursors: n = 4, cortical precursors: n = 6, vehicle: n = 4).

### Immunohistochemical Analyses

Six weeks after transplantation animals were euthanized with pentobarbital and perfused transcardially with PBS followed by 4% paraformaldehyde. Brains were postfixed overnight, equilibrated in graded sucrose and frozen in isopentane. Coronal 30 μm sections were cut serially on a freezing microtome (Microhm HM500). Immunohistochemistry was carried out on free-floating brain sections (in vivo studies) or directly on the culture dish (in vitro studies). The following primary antibodies were used: Monoclonal mouse antibodies: TH 1:100–250 (Chemicon International, Inc., Temecula, CA), GFAP 1:500 (Chemicon), CD68 kiM7 1:1,000 (Dako Immunochemicals, Carpinteria, CA), β-III tubulin 1: 400 (TuJ1, BabCo) Monoclonal rat: PSA-NCAM 1:200, (Pharmingen). Polyclonal rabbit: TH 1:100–300 (Pel Freeze Biologicals, Rogers, AR), doublecortin 1:200–1000 (a generous gift of C. Walsh), calretinin 1:200–1,000 and calbindin 1:200–1,000 (kindly provided by M. Celio). Single and double immunohistochemistry was carried out after permeabilization and blocking for unspecific binding with 0.3% Triton X-100 and 10% normal goat or donkey serum. Primary antibodies were incubated overnight at room temperature. Appropriate FITC or rhodamine linked secondary antibodies (both 1:100, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) or biotinylated secondary antibodies (1:250, Vector Laboratories, Burlingame, CA) followed by streptavidin-conjugated Texas Red (1:500, Jackson ImmunoResearch) were used for visualization. Tissue samples prepared for bright field microscopy were treated identically but incubated in 3% hydrogen peroxide for 15 min and visualized with 3-3′diaminobenzidine (Vector) and hydrogen peroxide. The sections were mounted on gelatin-coated slides, dried, dehydrated in ascending ethanol series, cleared in xylenes and mounted in Permount. Histological images were acquired with conventional Leitz and Zeiss Axiophot microscopes and with a confocal Zeiss Axiovert LSM 400 station. Estimation of TH+ cell numbers in the mesencephalic grafts was carried out using the optical fractionator technique (Gundersen et al., 1988) on a commercially available Stereology system (Stereo Investigator, Microbrightfield). Positive cells were counted under 400× magnification on every fourth uniform randomly chosen striatal section. An average of 90% of the grafted area was sampled with this method. Graft volume was calculated using Cavalieri estimation by point counting of every twelfth striatal section under 25× magnification. All results are expressed as mean ± SEM.

## RESULTS

### In Vitro Studies

In the presence of bFGF the total number of human mesencephalic and cortical precursors obtained at Day 7 of culture increased to an average of 6-fold the number of cells initially plated (Fig. 1A). BrdU incorporation studies confirmed ongoing cell proliferation in 54% and 47% of cortical and mesencephalic precursors respectively at Day 5 of culture (24 hr pulse). After 5 days of mitogen withdrawal in the presence or absence of a combination treatment with dbcAMP and AA mesencephalic precursors yielded 62% and 59% TUJ1+ neurons respectively. The percentages of neurons immunoreactive for TH were 21% in dbcAMP/AA treated and 12% in control cultures. Cortical precursors grown under identical conditions yielded 43% and 38% TUJ1+ neurons respectively. The percentages of neurons immunoreactive for TH+ in differentiated cortical precursors were 7.5% in dbcAMP/AA treated cultures and 2% in controls. All cultures used for grafting were differentiated for 2 days in the absence of bFGF (mitogen withdrawal) in medium supplemented with dbcAMP/AA. Supernatant collected from predifferentiated precursors during 15 min incubation in either HBSS (basal level) or HBSS containing 56 mM KCL (depolarization) was subjected to rpHPLC analysis for DA. Neurons derived from mesencephalic precursors yielded 75 ± 17 pg DA/ml under basal conditions and 154 ± 21 n = 4 after depolarization. DA levels in corresponding cortical precursor cultures were 23 ± 9 pg (basal) and 32 ± 12 pg DA/ml (depolarization), n = 4 (Fig. 1C).

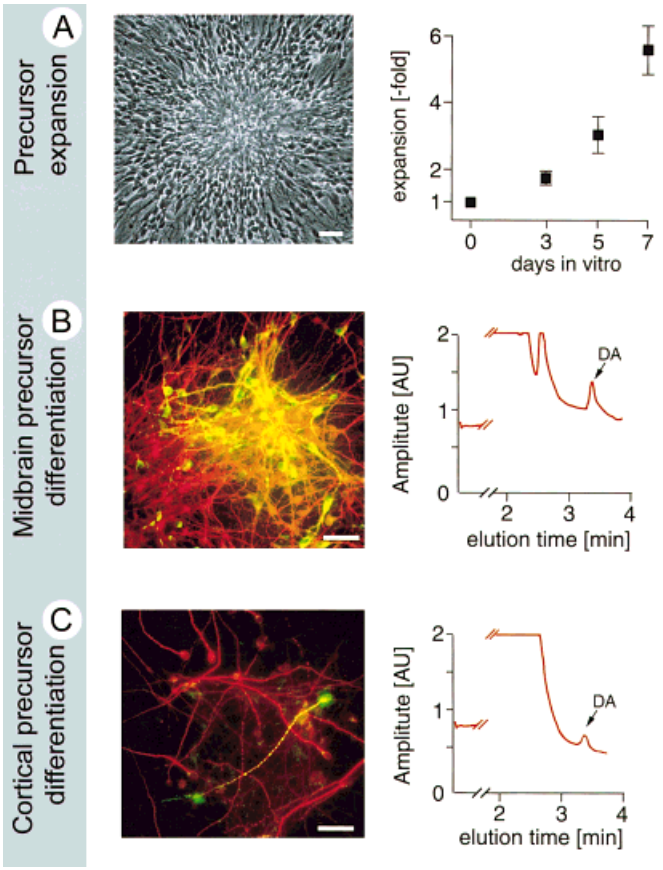


Fig. 1. In vitro analyses of mesencephalic and cortical precursors. **A:** Precursor cell expansion. **Left panel:** Phase contrast image of a large cell cluster derived from few single human midbrain precursors after 7 days of bFGF mediated in vitro proliferation. **Right panel:** The total cell number of human mesencephalic precursors showed an exponential increase over time in vitro. Data represent Mean +/ − SEM from quadruplicate experiments from a single embryo. **B:** Human mesencephalic precursor cell differentiation and in vitro function. Dopaminergic neurons were derived from mesencephalic precursors upon mitogen withdrawal and in the presence of dbcAMP and ascorbic acid (AA). **Left panel:** Immunohistochemistry for β-III tubulin (TUJ1, red, neuronal marker) and tyrosine-hydroxylase (TH, green, DA marker) revealed large numbers of TH/TUJ1 co-labeled neurons. **Right panel:** Representative rpHPLC chromatogram of KCL-evoked dopamine release in differentiated mesencephalic precursors. **C:** Human cortical precursor cell differentiation and in vitro function. Cortical precursor cells differentiated under identical conditions gave rise to TUJ1/TH (red/green) co-labeled cells (**left panel**) exhibiting DA release in vitro (**right panel**). The number of dopamine neurons was lower than in cultures derived from human mesencephalic precursors (see text). Scale bars = 50 μm.

### In Vivo Studies

Cells derived from human mesencephalic or cortical precursors were grafted into the ipsilateral striatum of rats with stable 6OHDA lesions (n = 6 cortical precursors; n = 4 mesencephalic precursors). Grafts were identified in vivo 6 weeks after transplantation by immunohistochemistry with a human specific antibody against neuron specific enolase (hNSE). Grafted cells could be readily detected in all but one animal (mesencephalic group). We excluded this animal from further analyses as complete absence of either graft or host reaction indicated a technical failure during cell implantation. Animals grafted with midbrain cells had smaller hNSE+ grafts (0.22 ± 0.01 $mm^3$, n = 3, Fig. 2A) than those with cortical transplants (0.56 ± 0.04 $mm^3$, n = 6). No immunoreactivity for hNSE was observed in the sham group. All animals tolerated immunosuppression without apparent side effects or signs of graft rejection. Only few GFAP+ cells were detected within the graft and immunoreactivity to CD68 was confined to few macrophages along the injection tracks and at the graft/host interface. Mesencephalic grafts were rich in TH+ cells (Fig. 2B, 953 ± 126 cells/graft, n = 3) although no significant numbers of TH+ cells were present in cortical grafts. The majority of grafted cells in both groups expressed calretinin (Figs. 2B, green) a neuron-specific calcium binding protein that is highly expressed during early CNS development (Gerfen et al., 1987; Fonseca et al., 1995). Some of the TH+ neurons in mesencephalic grafts co-expressed calretinin (Fig. 2B, yellow) but no calbindin or parvalbumin-expressing TH+ neurons were detected. In contrast the adult substantia nigra is composed of calretinin, calbindin and parvalbumin expressing dopamine neurons (McRitchie and Halliday, 1995). Cells exhibiting neuronal precursor characteristics such as immunoreactivity for PSA-NCAM and doublecortin were located outside mesencephalic graft borders in distances of up to 400 μm (Fig. 2C, Mes). Both PSA-NCAM and doublecortin were also strongly expressed in cortical grafts (Fig. 2C, C×) but immunoreactive cells were more densely packed within the core of the graft and only few cells were located outside the graft borders. Behavioral analysis shows a trend toward a decrease in amphetamine-induced circling response 6 weeks after transplantation in rats grafted with human mesencephalic precursors (−35%) but not in those grafted with either cortical cells (+13%) or vehicle control (−7%). The average rotation scores before and 6 weeks after transplantation were 10.2 ± 0.8 and 6.0 ± 1.1, n = 3 (mesencephalic precursors) and 9.9 ± 2.3 and 11.2 ± 1.5, n = 6 (cortical precursors), 10.3 ± 1.1 and 9.5 ± 1.5, n = 4 (vehicle control). Standard statistical methods (ANOVA interaction of group × time) did not yield statistically significant results. Decreased rotation scores were observed in all three rats with mesencephalic precursor grafts (>700 surviving TH cells each) but in none of the rats with cortical precursor or sham grafts (<100 TH cells each, n = 10). The small number of animals precludes definite conclusions about the behavioral data.

## DISCUSSION

Studies in rodents demonstrated that the successful in vitro derivation of DA neurons from dividing midbrain precursors is dependent on the developmental stage of the precursor cells (Studer et al., 1998). The stage that yields optimum DA neuron yield from rat midbrain precursors is embryonic day (ED) 11.5–12.5. We demonstrate efficient

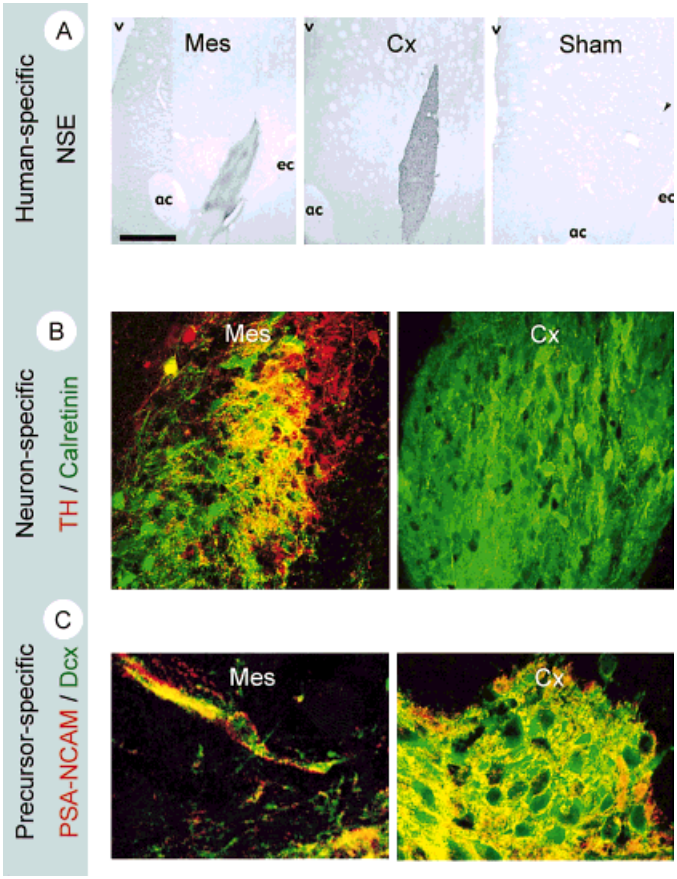


Fig. 2. Survival and neuronal differentiation of transplanted bFGF expanded human precursors. **A:** Identification of grafted human cells: grafts were identified using a human-specific antibody against neuronal specific enolase (hNSE). Mesencephalic grafts (Mes) were small and the grafted cells dispersed. Cortical grafts (Cx) exhibited strong immunoreactivity to hNSE and were clearly delimited from the host striatum. No hNSE+ cells were present in sham-grafted animals (Sham). A small group of macrophages (arrow), barely recognizable at low power, identifies the infusion tract. **B:** Neuronal subtype characterization: Confocal images of TH (red) and calretinin (green) immunoreactivity: Only mesencephalic grafts contained TH+ cells although calretinin was highly expressed in both mesencephalic and cortical grafts. Calretinin/TH co-expression was detected in a proportion of the grafted mesencephalic precursors. **C:** Precursor cell characterization. Confocal images of PSA-NCAM (red) the polysialated form of the neural cell adhesion molecule, and doublecortin (green) a marker of immature neurons expressed during cell migration. These two markers were highly expressed in both mesencephalic and cortical grafts. In mesencephalic grafts doublecortin was predominantly localized in the periphery of the graft and cells leaving the graft core showed co-localization of both markers. In cortical grafts the majority of cells expressed doublecortin and most of them co-localized PSA-NCAM. Legend: ac, anterior commissure; ec, external capsule; v, lateral ventricle; Scale bar in A = 1,000 μm in (A), 100 μm in (B) and 20 μm in (C, Mes) and 25 μm (C, Cx).

DA neuron differentiation from human mesencephalic precursors derived from an embryo 7 weeks p.c. Cortical precursors derived from this early developmental stage could also be differentiated into DA neurons in vitro. No surviving cortex-derived DA neurons could be detected in vivo 6 weeks after grafting and no changes in behavioral parameters were observed. Primary human fetal mesencephalic tissue grafted into the striatum of hemiparkinsonian rats typically requires up to 18 weeks for improving behavioral parameters in these animals (Brundin et al., 1988). Our data show a trend toward behavioral improvement 6 weeks after transplantation of pre-differentiated human mesencephalic precursors. Larger cohort and long-term studies are now clearly needed to systematically compare behavioral and histological results with primary human fetal tissue.

Transplanted cells within the graft expressed several precursor cell markers such as PSA-NCAM (Rousselot et al., 1994; Hu et al., 1996) and doublecortin (Francis et al., 1999; Gleeson et al., 1999). The graft cytoarchitecture of PSA-NCAM and doublecortin+ zones was reminiscent of the immunohistochemical organization of the adult SVC suggesting that in vivo development of precursor cell grafts might mimic some aspects of normal CNS development (data not shown). Previous studies showed that calbindin and calretinin are first expressed in an ascending caudo-rostral gradient reaching the cerebral vesicles by 6–7 week p.c. (Hof et al., 1999). In rodents calretinin+ neurons are generated first in the ventral tier of the substantia nigra (Gerfen et al., 1987) and often precede the expression of calbindin during brain development (Yan et al., 1995). It is tempting to speculate whether selective expansion of early precursor cell pools, yielding calretinin+ cells, could account for the predominance of calretinin+ cells and the absence of any cells co-expressing TH and calbindin or parvalbumin in vivo.

The majority of surviving cells in both mesencephalic and cortical grafts were neurons. This result contrasts with previous transplantation studies using undifferentiated CNS precursors that reported primarily glial differentiation (Svendsen et al., 1996; Winkler et al., 1998; Fricker et al., 1999). The most likely explanation for these differences is the pre-differentiation phase in our study that allows undifferentiated precursors to establish stable neuronal or dopaminergic phenotypes before transplantation. In agreement with this hypothesis, a recent study demonstrated that DA cells in primary nigral grafts differentiate before implantation (Sinclair et al., 1999).

Further improvements in our ability to generate human dopamine neurons can be envisaged based on the growing molecular understanding of cell fate determination (Hynes and Rosenthal, 1999) and the successful derivation of dopamine neurons from alternative cell sources such as embryonic stem (ES) cells as shown recently for mouse cells (Kawasaki et al., 2000; Lee et al., 2000. Our results suggest that in vitro proliferation of CNS precursors is a promising alternative to human fetal tissue and encourage further detailed quantitative studies addressing clinical potential.

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