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The Potential for Circuit Reconstruction by Expanded Neural Precursor Cells Explored through Porcine Xenografts in a Rat Model of Parkinson's Disease

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Neural precursors with the properties of neural stem cells can be isolated from the developing brain, can be expanded in culture, and have been suggested as a potential source of cells for neuronal replacement therapies in degenerative disorders such as Parkinson's disease (PD). Under such conditions an improved spectrum of functional benefit may be obtained through homotypic reconstruction of degenerated neural circuitry, and to this end we have investigated the potential of expanded neural precursor cells (ENPs) to form long axonal projections following transplantation in the 6-hydroxydopamine-lesioned rat model of PD. ENPs have been isolated from the embryonic pig, since implantation in a xenograft environment is thought to favor axonal growth. These porcine ENPs possessed similar properties *in vitro* to those described in other species: they proliferated in response to epidermal and fibroblast growth factor-2, expressed the neuroepithelial marker nestin, and differentiated into neurons, astrocytes, and occasional oligodendrocytes on mitogen withdrawal. The use of pig-specific markers following xenotransplantion into cyclosporin A-immunosuppressed rats revealed that many cells differentiated into neurons and displayed extensive axogenesis, such that when placed in the region of the substantia nigra fibers projected throughout the striatal neuropil. These neurons were not restricted in the targets to which they could project since following intrastriatal grafting fibers were seen in the normal striatal targets of the pallidum and substantia nigra. Staining for a pig-specific synaptic marker suggested that synapses were formed in these distant sites. A small number of these cells differentiated spontaneously to express a catecholaminergic phenotype, but were insufficient to mediate behavioral recovery. Our results suggest that when the efficiency of neurochemical phenotype induction is increased, ENP-derived neurons have the potential to be a uniquely flexible source of cells for therapeutic cell replacement where anatomical reconstruction is advantageous. © 2002 Elsevier Science (USA)

Key Words: **neural stem cell; EGF; FGF-2; xenotransplantation; cell therapy; tyrosine hydroxylase.**

INTRODUCTION

Cell replacement therapy through transplantation of dopaminergic neuroblasts isolated directly from the developing midbrain of human embryos can yield significant therapeutic benefit in patients with Parkinson's disease (PD) (23, 41, 77). However, current procedures are problematic: the use of multiple human embryos presents practical and ethical difficulties and development of effective alternatives is imperative if cell replacement therapy is ever to be broadly applicable. Furthermore, the delivery of tissue into the ectopic target site of the caudate-putamen does not appear to be compatible with complete symptomatic relief and may (in rare circumstances if the implantation site is not precisely controlled) lead to serious side effects (29).

This is reminiscent of the situation in animal models where intrastriatal dopaminergic grafts fail to ameliorate certain lesion-induced functional deficits, even when extensive striatal reinnervation is achieved (14, 25, 26, 79). It has been proposed that a fuller spectrum of functional recovery, and an improved side effects profile, may result from placement of grafts in the substantia nigra (SN) and subsequent homotypic circuit reconstruction (6, 79). The ectopic placement, however, is used out of necessity as animal work has clearly shown that homotopic, intranigral allografts (e.g., rat to rat) mediate no major functional benefit

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due to the fact that fibers remain confined to the graft mass and do not project along the inhibitory white matter of the mature nigrostriatal tract to reinnervate the striatal neuropil (2, 9, 24, 78). In contrast, xenografted tissue (e.g., pig to rat; human to rat) appears to be able to overcome this inhibition and displays profuse axon outgrowth to project long distances to target sites specific for its region of origin (21, 32, 32, 75). Thus, neurons from intranigral pig-to-rat or human-to-rat primary ventral mesencephalic xenografts project in the nigrostriatal tract to reinnervate the striatum, although the functional status of these grafts has not been assessed (35, 76).

It has been suggested that multipotential neural stem cells might constitute an ideal renewable source of tissue for neural transplantation therapy (4, 10, 31, 61). Populations enriched for such cells can be expanded *in vitro,* using the mitogens epidermal and fibroblast growth factors, from the brains of developing and adult animals as cultures in suspension ("neurospheres") or attached to a substrate (51, 52, 72). Our use of the generic term *expanded neural precursors* (ENPs) to describe these cells emphasizes the fact that these mixed populations are composed of stem cells and lineage-restricted precursor cells derived from them. In some studies (59, 62), but not others (45, 63, 70), such cells have been shown to differentiate into tyrosine hydroxylase-positive neurons following ectopic intrastriatal transplantation in rat models of PD and may mediate some functional effects (albeit variable and incomplete). In the current study, the advantages of the neural xenograft paradigm described above have been exploited to investigate the ability of ENPs to differentiate into projection neurons following transplantation and their potential to reconstruct the nigrostriatal circuitry disrupted in PD.

MATERIALS AND METHODS

Isolation and Expansion of Porcine Expanded Neural Precursors (pENPs)

Pig embryos of gestational age E26–27 (crown to rump length 17.5–18.5 mm) were used (n = 5 litters). The normal gestation of the pig is 115 days, and the stage used corresponds to Carnegie stages 18–19 (Fig. 1a). Embryos were removed by sterile hysterectomy from pregnant sows (Large White, Imutran ltd.), which had been killed with Lethobarb (pentobarbital sodium, 200 mg/ml (20%)). Following removal of the brain, the cerebral cortex was dissected in ice-cold sterile phosphate-buffered saline (PBS, pH 7.4) supplemented with 0.6% glucose. Tissue was digested in PBS containing trypsin (240 U/ml, Worthington), 0.001% DNase (Sigma), and 0.6% glucose for 20 min at 37°C; washed 3× in PBS, 0.001% DNase, 0.6% glucose; and dissociated mechanically to a coarse single cell suspension, with cell number and viability being determined by trypan blue exclusion. Populations of growth-factor-responsive neural precursors were prepared as described previously for human precursors (5, 65). Briefly, cells were seeded at a density of 200,000 viable cells per milliliter in medium composed of DMEM/Ham's F12 (1:3, Invitrogen) supplemented with B27 (2%, Invitrogen), penicillin/streptomycin/fungizone (PSF, 100 μg/ml, Invitrogen), EGF (human recombinant, 20 ng/ml, Sigma), FGF-2 (human recombinant, 20 ng/ml, R&D Systems), and heparin (5 μg/ml, Sigma). Proliferating precursors were fed every 4 days by replacing half the medium with fresh. Cells grew in suspension as spheres that reached a diameter of 300–400 μm by 13–14 days (Fig. 1b), at which point they were harvested by centrifugation and the supernatant was removed. Spheres were placed onto the lid of a plastic petri dish and sectioned using a MacIlwain tissue chopper set at 250 μm (65). Chopped pieces were resuspended in 1 ml of proliferation medium, and cell number and viability were determined by dissociating an aliquot of the chopped sphere suspension. Chopped pieces were then reseeded at a density of approximately 200,000 cell/ml in a proliferation medium that was identical to that used initially save for the substitution of B27 with N2 (1%, Invitrogen). Cells were prepared for use in the experiments described below 10–14 days following this procedure, by which point spheres had again attained 300–400 μm in diameter.

Examination of pENP Differentiation in Vitro

The phenotypic fate of pENPs was assessed *in vitro* following dissociation of the spheres to a coarse single-cell suspension, using a trypsin digest and mechanical trituration as described above. Cells were then plated onto 13-mm poly-L-lysine-coated glass coverslips at a density of 50,000 cells in 30 μl of "differentiation medium" composed of DMEM, 2% B27, 1% PSF, and 1% fetal calf serum (Harlan). After 30–60 min, cells had attached to the substrate and were either immediately fixed by flooding with 10% formalin (20 min followed by three washes in PBS) or flooded with 500 μl of differentiation medium and placed in a humidified incubator at 37°C, 5% CO_2 prior to fixation. Cells were fed by replacing half the medium with fresh every 3 days. BrdU labeling was achieved by adding BrdU (10 μg/ml, Boehringer) to an aliquot of the pENP cultures for 20–22 h prior to dissociation and plating. Prior to BrdU staining, coverslips were postfixed in methanol for 20 min at −20°C, washed three times in PBS, incubated with 2 M HCl for 20 min at 37°C, washed twice in 0.1 M sodium borate, and washed again in PBS. Indirect fluorescent immunocytochemistry was performed using standard protocols with primary antibodies directed against nestin (1:25, a generous gift of Dr. R. McKay), β-III-tubulin (TuJ1, 1:750, Sigma),

GFAP (1:1000, DAKO), galactocerebroside (GalC, 1:3, a generous gift of Professor N. Scolding), tyrosine hydroxylase (TH, 1:400, Sigma), 32-kDa dopamine- and adenosine-related phosphoprotein (DARPP-32, kind gift of Dr. Ouimet), and BrdU (1:300, Boehringer). These protocols have been described in detail previously (5, 64) Staining was visualized on a Leitz DRMB microscope, and cell counts were performed at ×40 magnification using a grid. Five randomly selected fields from two replicates (coverslips) of each of three to five independent cultures were counted. Pseudocolor fluorescent images were obtained using Openlab 2.1 image analysis software.

Animals

Female Sprague–Dawley rats (Charles River, UK) weighing 200–240g at the start of the experiment were used. Animals were housed in a natural light–dark cycle and were allowed access to food and water (supplemented with the antibacterial agent terramycin (Pfizer)) *ad libitum* throughout the course of the experiment. Animal experiments were performed in compliance with the UK Animals (Scientific Procedures) Act 1986.

6-Hydroxydopamine (6-OHDA) Lesions and Amphetamine-Induced Rotation

All surgery was performed under halothane (Fluothane) anesthesia. Animals received unilateral 6-OHDA lesions of the right medial forebrain bundle. Each animal received pargyline 30–60 min prior to surgery (Sigma, 50 mg/kg/ml in normal saline, ip) followed by 4 μl of 6-OHDA · HBr (2 μg/μl, Sigma, free base dissolved in 0.9% saline/0.2% ascorbate) infused over a 4-min period using a 10-μl Hamilton syringe targeted at stereotaxic coordinates −4.4mm anterior (A) to bregma, −1.0 mm lateral (L) to bregma, and −7.8 mm below dura (vertical, V), with the incisor bar set to 2.3 mm below the interaural line. The syringe needle was left in place for 2 min after the infusion to allow for diffusion of the toxin. The wound was then cleaned and sutured.

Rotational behavior was assessed in a bank of automated rotometer bowls after Ungerstedt and Arbuthnott (68). Amphetamine-induced rotation was measured as the number of full turns over 90 min following administration of methamphetamine sulfate (Sigma, 2.5 mg/kg dissolved in 0.9% saline, ip) and was carried out twice prior to grafting. Only animals that had mean net rotations of >5 turns/min were included in the experiment. Animals were matched on these scores for allocation to the experimental groups detailed below and were tested fortnightly, beginning 3 weeks after transplantation.

Neural Transplantation

An estimate of the cell number and viability within the pENP sphere suspension was obtained from two aliquots of the suspension which were dissociated, via a trypsin digest and mechanical trituration, to single cells and counted under a hemocytometer using trypan blue exclusion. Using this information, undissociated pENP spheres were harvested by centrifugation and resuspended in a grafting medium composed of DMEM, at approximately 250,000 viable cells per microliter. Over a period of 2 min, 2 μl (approx. 500,000 cells) was delivered stereotaxically to either the striatum (A +0.6 mm, L −2.4 mm, and V −4.5 mm) or the mesencephalon at the level of the substantia nigra (A −4.6 mm, L −2.8 mm, and V −7.0 mm) of lesioned rats, using a needle of bore diameter 0.25 mm (SGE Chromatography). The needle was left in place for 2 min following grafting to minimize reflux of grafted cells along the needle tract following which the needle was removed and the incision was closed and sutured. Animals received daily injections of CsA (Sandimmun, Novartis, 10 mg/kg, ip), for the duration of the experiment, commencing the day prior to transplantation.

Histology and Immunocytochemistry

Animals were transcardially perfused, under terminal barbiturate anesthesia, with 100 ml of PBS (pH 7.4) followed by 250 ml of 4% paraformaldehyde (in PBS, pH 7.4) 9 weeks after transplantation. Brains were left in 4% paraformaldehyde overnight, transferred to 25% sucrose until they sank, and then sectioned at 60 μm on a freezing stage microtome. Standard histological protocols were used to stain a 1:6 series of sections for Nissl substance using cresyl fast violet, after mounting onto gelatinized slides. Immunocytochemistry was performed at room temperature on freely floating sections to maximize antibody penetrance. The following primary antibodies were used: pig-specific 70-kDa neurofilament (NF_{70}, 1:2000, Dr. Luis Soriano), TH (1:4000, Jacques-Boy Institute), NeuN (1:500, Chemicon), pig-specific synaptobrevin (1:500, Chemicon), vimentin (1:500, Sigma), CD8 (1:500, Serotec), and rat IgM (1:500, Sigma). The protocol has been described in detail previously (8). Staining controls in which the primary antibodies were omitted from the protocol confirmed the specificity of staining. Use of the pig-specific antibodies for NF_{70} and synaptobrevin on sections of ungrafted rat brains confirmed that this antibody did not cross-react with rat isoforms of the protein.

Cell Counts and Statistical Analysis

Graft volume was determined with reference to a 1:6 series of cresyl fast violet stained sections. Area mea-

surements were obtained by tracing around a digitized image of the graft (Seescan, UK) and were then used to obtain volume estimates in cubic millimeters by taking into account the section thickness and frequency.

The relatively small numbers of TH-immunopositive cells allowed all of these cells to be counted on a 1:6 series through the graft, and total numbers were estimated and corrected after Abercrombie (1). A stereological analysis system was used to obtain an unbiased estimate of the total number of NeuN-immunopositive cells within the grafts. This consisted of an Olympus BX50 microscope with an *x–y* motorized slide platform that was interfaced to a computer running Olympus CAST GRID v.2.0. This software package was used to obtain cell counts by randomly and systematically placing a counting frame over the delineated graft area, and total cell numbers were extrapolated using a stereological algorithm (73). The soma volume of NeuN-positive cells that were present within the presumptive graft mass was measured. This analysis aimed to determine whether porcine origin of these neurons within the presumptive graft mass could be confirmed due to the larger size of pig neurons with respect to host rat neurons. Central sections from six representative grafts were analyzed (three intrastriatal and three intramesencephalic), and 12 immunopositive cells from each graft were selected randomly using the automated stereological sampling protocol (Olympus CAST GRID). For comparison, in each brain, a region was demarcated in the contralateral host brain that was identical in area and location to the grafts, and 12 host NeuN-positive cells were analyzed. To determine soma volume, seven independent volume estimates were obtained on each cell using the optical dissector (Olympus CAST GRID), and a mean was obtained.

Striatal innervation by NF_{70}-expressing fibers was estimated by counting immunopositive fibers in the dorsal and ventral striatum. Anatomical landmarks were used to select, define, and standardize the location of counting frames of a set size (illustrated in Fig. 7): (a) dorsal striatum (rostral) defined by joining of the corpus callosum; (b) dorsal striatum (caudal) defined by crossing of the anterior commissure; and (c) ventral striatum at the level of the nucleus accumbens. The number of discrete fibers present in each frame was counted and expressed as fibers per square millimeter.

Multifactorial analysis of variance was performed using the Genstat 5 release 3.2 statistical package (Lawes Agricultural Trust, Rothamstead Experimental Station, UK). Post hoc comparisons were with the Newman–Keuls test. Chi-squared analyses were performed by hand. In all instances a probability of <0.05 was considered statistically significant.

RESULTS

Expansion and Differentiation of Porcine Neural Precursor Cells in Vitro

A combination of EGF and FGF-2 was effective for inducing the expansion of pENPs isolated from the E26/27 cerebral cortex (Fig. 1a). Cells proliferated as clusters of undifferentiated cells, similar in appearance to the neurospheres described for other species (Fig. 1b). These pENPs could be propagated using techniques that have been optimized for the rapid, long-term expansion of human neural precursors (65) and allowed for an 8.3 ± 1.3-fold ($n = 5$) increase in total cell number to be obtained 23–25 days following isolation. At this time point, clusters could be dissociated and pENPs induced to differentiate by withdrawing the mitogens and exposing cells to an inert polycationic substrate. Thirty minutes after plating, cells retained an immature morphology, and over 90% expressed the neuroepithelial precursor cell marker nestin that had a cytoplasmic, filamentous distribution (Fig. 1c), while few cells expressed markers for differentiated neuronal or glial phenotypes (TuJ1 5%, GFAP 0%, GalC 0%, not shown). Supplementing the growth medium with BrdU 20 h prior to plating resulted in nuclear labeling of nestin-positive cells (Fig. 1c). By 7 days pENPs differentiated into mixed populations of TuJ1-immunopositive neurons (around 40%), GFAP-positive astroglia (around 15%), and occasional GalC-positive oligodendroglia (less than 1%) (Fig. 1e); other cells were not stained by any of these markers (Fig. 1d). Immunocytochemistry for NF_{70} and MAP2ab at this time confirmed that a mature neuronal phenotype had been adopted (not shown), which was also reflected by extensive neuritic outgrowth and complex morphologies of differentiated neurons (Fig. 1d). No TH-positive neurons, however, were observed *in vitro* at any time point but GABA immunochemistry revealed that 45% of the neurons were of this phenotype (Fig. 1f).

Survival of Transplanted pENPs

In order to assess the potential of porcine cortical neural precursor populations to survive neural xenotransplantation, and their capacity to differentiate into therapeutically useful phenotypes *in vivo*, pENPs were grafted into the corpus striatum or mesencephalon (in the region of the substantia nigra) of cyclosporin A-immunosuppressed, 6-OHDA-lesioned rats. All animals (15/15) had large, healthy, surviving grafts 9-weeks after transplantation, which were easily identified on sections stained for Nissl substance (Figs. 2a and 2b). Discrete graft masses containing cells with characteristic neuronal and glial profiles were apparent and there were no gross histological differences between grafts implanted into the striatum and those implanted into the mesencephalon, save that there was

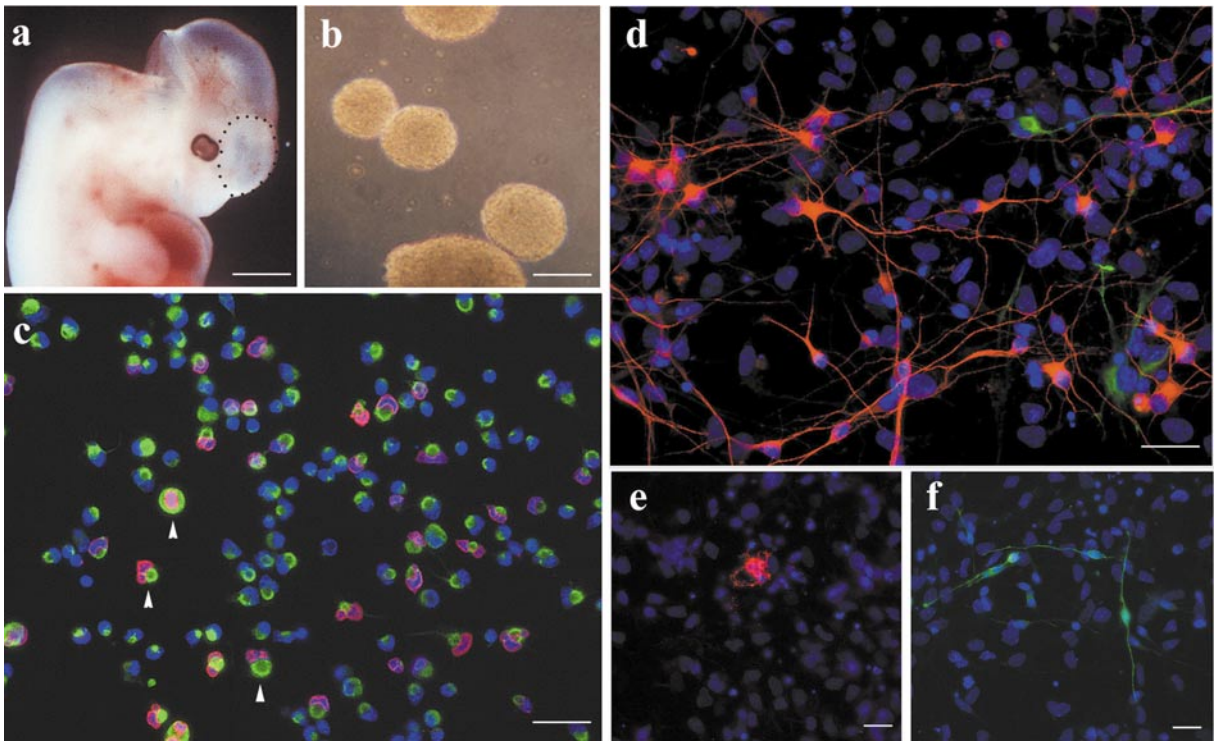

FIG. 1. Properties of pENPs *in vitro.* (a) Photograph illustrating the appearance of the E26 pig embryo and the dissected region of cerebral cortex (scale 1 mm). (b) The appearance of pENPs immediately prior to grafting and 1 week after passaging (scale 200 μm). (c) Photomicrograph of pENPs (26 days of growth) which were dissociated, plated, and fixed after 30 min; BrdU was added to this culture 20 hr prior to plating. Green FITC labeling shows that the majority of cells are immunopositive for nestin; red RITC labeling is for BrdU. All BrdU-labeled cells are nestin positive, and mitotic figures are apparent in several cells (arrowheads) (scale 100 μm). (d–f) pENPs differentiated for 7 days *in vitro:* in (d) cells are stained for TuJ1 (RITC) and GFAP (FITC); in (e) cells are stained for GalC; and in (f) cells are stained for GABA. (d, scale 100 μm; e, f, scale 50 μm.)

a tendency for intramesencephalic grafts to be slightly larger, although this trend did not reach statistical significance (Table 1). In no case was there any evidence of a host-mediated rejection response; specifically, no cellular infiltrate, perivascular cuffing nor vascular engorgement was observed in any case. This minimal host immune response to grafted pENPs was confirmed by a virtual absence of immunocytochemical labeling for rat CD8-positive cells and rat IgM antibodies (Figs. 2c and 2d), the presence of which correlates with the rejection of intracerebral xenografts (3, 8, 48).

Phenotype of Transplanted pENPs

Expression of NeuN, the neuron-specific nuclear protein (44), and of vimentin, a marker for immature and reactive astrocytes (7, 17), was examined in order to assess the phenotypic fate of transplanted pENPs.

NeuN-immunoreactive neuronal cell bodies were distributed throughout both intrastriatal and intramesencephalic grafts (Figs. 3a and 3e). Within the graft mass, NeuN-positive neurons were larger and more immunoreactive than surrounding host neurons. To confirm the donor origin of these cells neuronal soma volume was measured and compared with that of host neurons that were present in the same anatomical location on the contralateral (untransplanted) side (36). Soma volume in the putative graft mass was on average 75% greater than contralateral neurons, irrespective of whether they were implanted into the striatum or mesencephalon (Fig. 4a, $\chi^2_{14} = 66.07$, $P < 0.001$), thus confirming the donor origin of these cells.

The adoption of a neuronal fate by donor pENPs was further confirmed by immunochemistry for the pig-specific isoforms of NF_{70} and synaptobrevin (see below). Quantification revealed a nonsignificant trend for intramesencephalic grafts to contain more neurons ($t_{13} = 2.17$, ns, Table 1). In both sites, NeuN-positive cells predominantly adopted an ovoid morphology, although large pyramidal-like cells were occasionally seen (Figs. 4c–4h). Subjectively, there were no morphological differences between neuronal populations present in grafts in the two sites and quantitative analysis revealed no significant differences between neuronal soma volume (Fig. 4b, $\chi^2_{12} = 10.67$, ns).

Large numbers of vimentin-positive cells with a fibrous astrocytic morphology were present throughout the graft parenchyma in all animals. There were no obvious differences between grafts in the two sites,

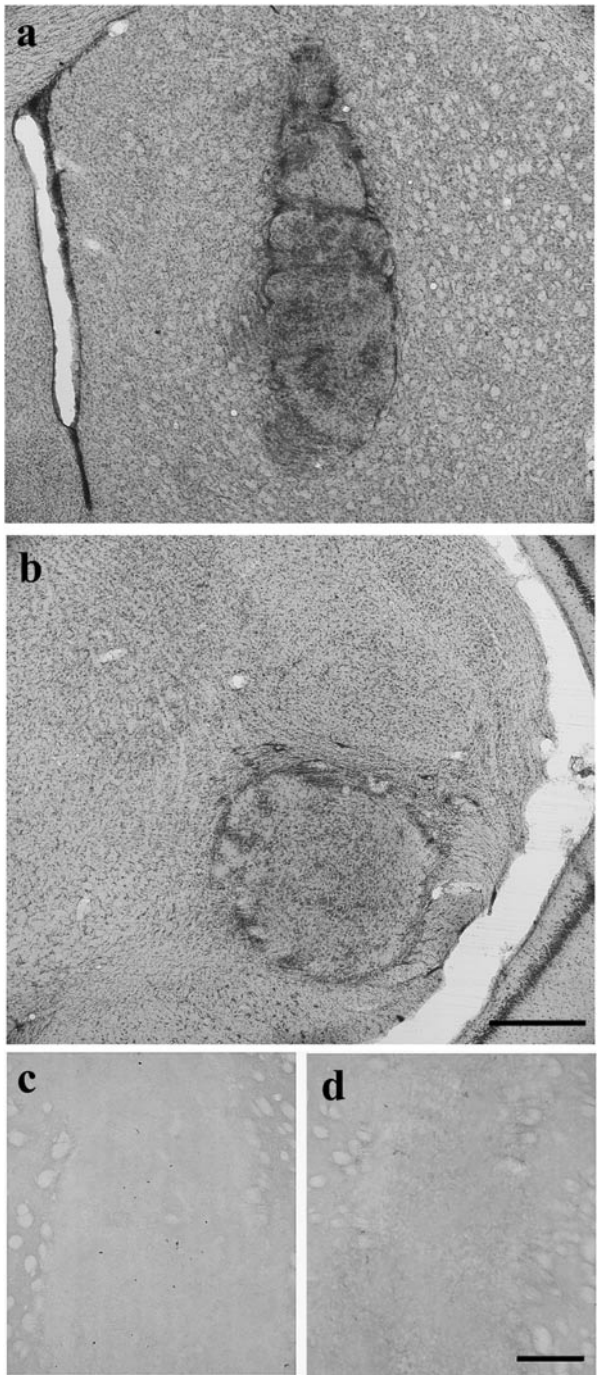

FIG. 2. Survival and morphology of pENP grafts after transplantation. (a and b) Intrastriatal and intramesencephalic grafts, respectively, stained for Nissl substance using cresyl fast violet; there are no apparent histological differences between grafts in the two sites and no gross signs of rejection (scale 1000 μm). (c) A representative intrastriatal graft stained immunochemically for CD8; a few sparsely distributed cells are apparent at the level seen in nonrejecting isografts (8). The lack of IgM binding to the same graft in (d) further indicates that this graft has failed to recruit a significant host immune response (c, d, scale 750 μm).

TABLE 1

Parameters of Intrastriatal and Intranigral Grafts of pENPs

	Intrastriatal	Intranigral
Graft volume (mm^3)	1.11 ± 0.13	1.71 ± 0.43, ns
NeuN total ($\times 10^5$)	2.6 ± 0.3	4.8 ± 1.0, ns
TH total	191.6 ± 55.2	57.0 ± 28.1*
% TH (estimate)	0.09	0.02

Note. Although there is a trend for intranigral grafts to be larger and contain more neurons (as assessed by stereological counts of NeuN-stained neurons) than intrastriatal grafts, it does not reach the level of statistical significance. Significantly more precursors differentiate to express TH when grafted to the striatum (Student's t test, $t = 2.33$, $P < 0.05$). An estimate of the proportion of total neurons which express TH has been obtained by expressing the TH count as a percentage of the NeuN count.

although the fibrous nature of the stain precluded their accurate quantification. In addition, a rim of densely immunoreactive cells surrounded the periphery of all grafts, which most likely represented a host astrocytosis (Figs. 3b and 3f) (7). In lesion-only control brains, vimentin immunoreactivity was largely confined to rounded cells in the ependymal layer surrounding the lateral ventricle and few, lightly stained cellular processes in myelinated fiber tracts in the ipsilateral striatum (not shown).

Since a principal aim of this study was to determine whether pENPs could adopt phenotypes that might be functionally useful in this PD model, TH immunoreactivity was examined. Small numbers of TH-immunopositive neurons were present in both intrastriatal and intramesencephalic grafts. In both locations, TH-positive cells were distributed evenly throughout the body of the graft mass and accounted for a small proportion of the total neuronal population (Table 1). Cell counts revealed that significantly more pENPs differentiated to express TH in the intrastriatal grafts (Table 1, $t_{13} = 2.33$, $P < 0.05$) although the magnitude of this effect was small. TH-positive neurons often showed complex morphologies (Figs. 3c, 3d, 3g, and 3h), although there was no evidence that processes from these cells projected across the graft–host border. To assess whether intrastriatally grafted ENPs had adopted a neurochemical phenotype characteristic of striatal projection neurons, selected sections were stained for DARPP-32. No immunopositive cells were seen.

Fiber Outgrowth and Striatal Reinnervation by pENPs

Immunohistochemistry for pig-specific NF_{70} allowed the fiber projections of the pENP-derived neurons to be mapped. A dense plexus of fibers was seen throughout the graft, irrespective of the graft site (Figs. 5a and 5e). Large numbers of fibers clearly traversed graft–host

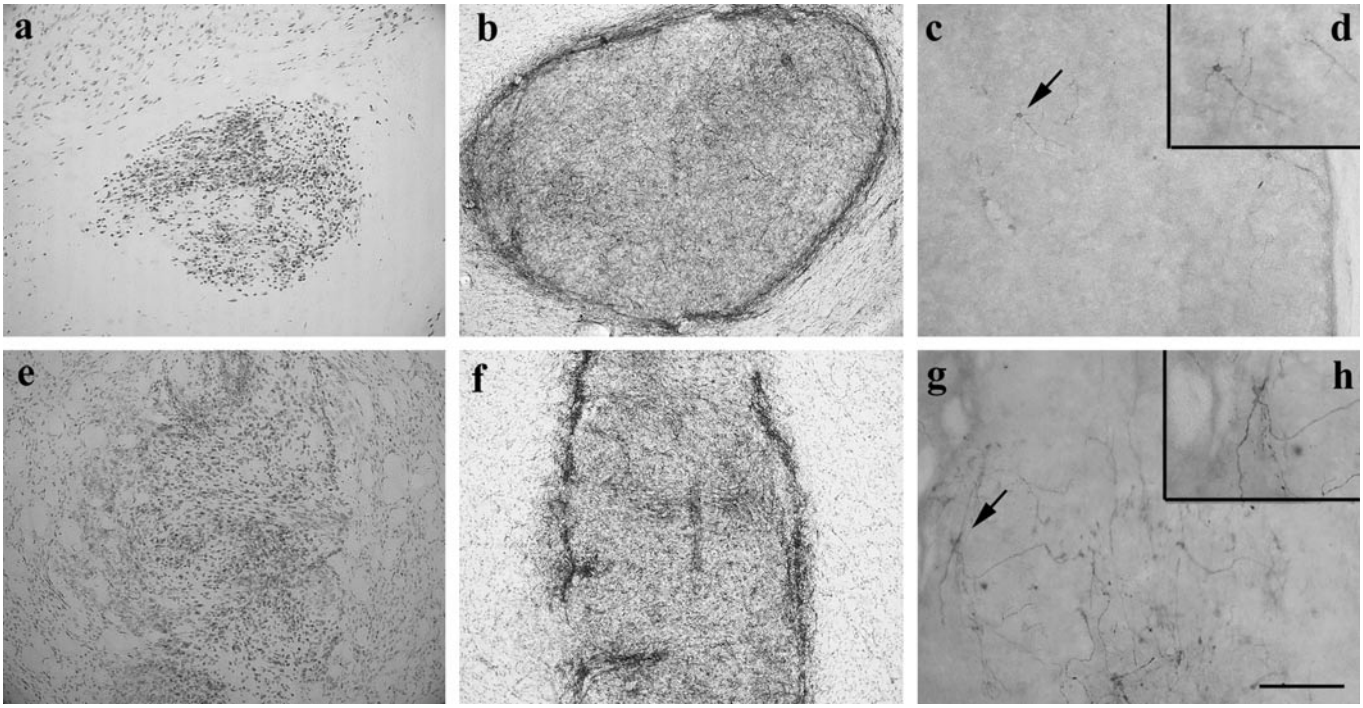

FIG. 3. pENP grafts contained both neuronal and glial phenotypes. (a–d) Intramesencephalic grafts stained for (a) the neuronal nuclear marker NeuN; (b) the astrocyte marker vimentin with a hematoxylin counterstain. (c) TH immunohistochemistry with (d) showing the arrowed detail of (c) to illustrate the morphological maturity of some TH-positive neurons. (e–h) Intrastriatal grafts stained for: (e) NeuN; (f) vimentin with a hematoxylin counterstain; (g) TH; (h) the arrowed detail of (g). The proportion of pENPs differentiating into these phenotypes did not differ markedly between the two sites (Table 1) (scale 300 μm).

borders to enter both the adjacent host neuropil and the myelinated fiber tracts (Figs. 5b and 5f). NF_{70}-positive fascicles projected very considerable distances along these tracts and were frequently seen to exit and ramify within host gray matter, including target sites appropriate for the site of implantation. Intramesencephalic pENPs projected rostrally along the medial forebrain bundle and the internal capsule and ramified within the mediodorsal thalamus, the caudate-putamen (CPu), the ventral striatum, and amygdaloid nuclei (Figs. 5g and 5h). NF_{70}-positive fibers from intrastriatal pENPs arborized directly within the gray matter of the CPu. They were also seen to course caudally along internal capsule fibers (where many were seen to exit and branch in the pallidum (Fig. 5c) and entopeduncular nucleus) and were also apparent in the external capsule and substantia nigra pars reticulata (Fig. 5d). Subsequent staining of sections that had received pENP grafts showed that in gray matter areas where fibers were seen to ramify, punctate immunoreactivity for pig-specific synaptobrevin (20) was demonstrable, suggesting that pENP-derived neurons were forming synapses with the host (Fig. 6).

As a principal aim of this study was to investigate the potential of pENPs to reconstruct degenerate circuitry and reinnervate the striatal neuropil following the 6-OHDA lesion, a method was devised which allowed the degree of striatal reinnervation to be assessed quantitatively (Fig. 7). This revealed significant differences in the striatal innervation by intramesencephalic and intrastriatal grafts (graft site $\times$ innervation site, $F_{2,30} = 6.80$, $P < 0.01$) (fig. 7). Intramesencephalic grafts projected similarly throughout the extent of the host striatum, with no significant differences in the degree of innervation at different levels. Intrastriatal grafts yielded a significantly greater fiber density than intramesencephalic grafts in the rostral and caudal dorsal striatum (post hoc Newman–Keuls; intramesencephalic $\times$ intrastriatal; anterior and posterior both P & lt;0.01), but not in the ventral striatum which was innervated to the same extent by grafts in both sites.

Amphetamine-Induced Rotational Behavior

There was no significant reduction in amphetamine-induced rotational behavior in either group with respect to the controls nor with respect to pregraft values at any time point (Time $F_{4,56} = 1.41$, ns; Graft $\times$ Time $F_{8,56} = 1.49$, ns).

DISCUSSION

In the current study expanded neural precursor cells from the developing pig brain were transplanted into

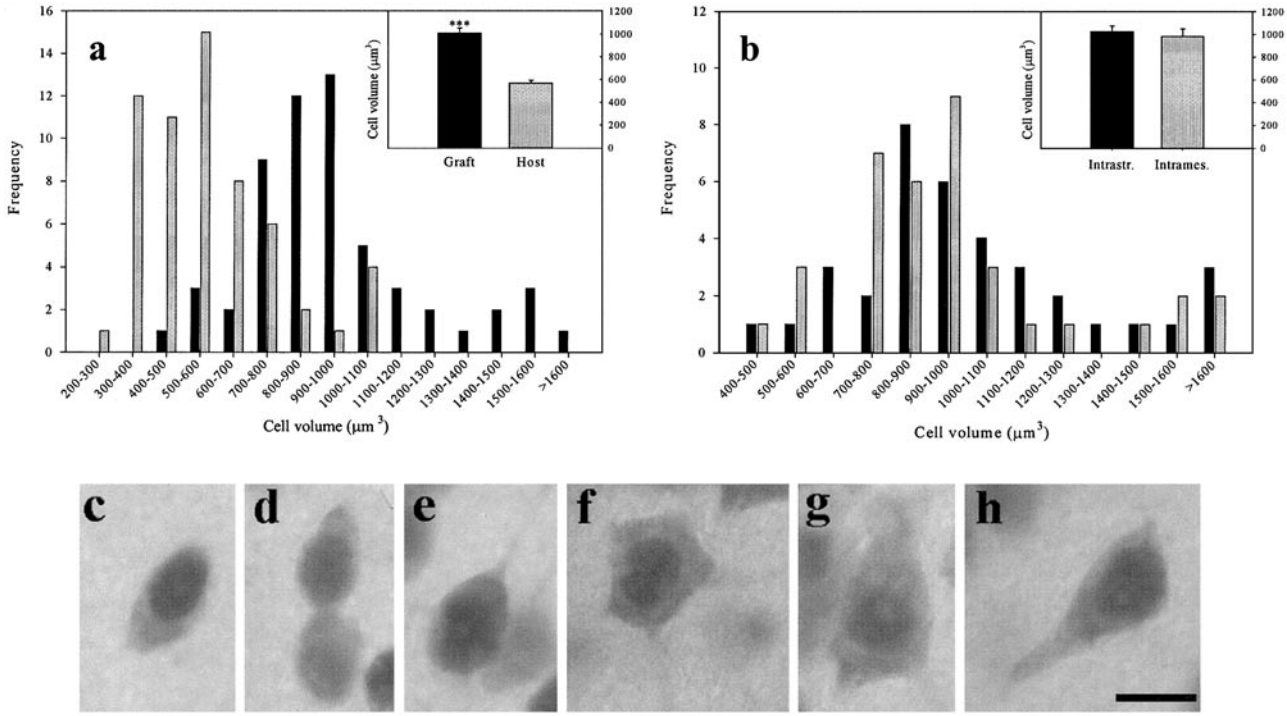

FIG. 4. (a) Neuronal soma volume of NeuN-immunoreactive cells within the presumptive graft mass (black bars) and an anatomically identical region on the contralateral, ungrafted side of the host brain (gray bars). Both intramesencephalic and intrastriatal grafts are included in this analysis. A frequency distribution histogram is shown and the peaks of these two populations are significantly different ($\chi^2_{14} = 66.07$, $P < 0.001$). The mean (+SEM) value is shown and indicates that the mean soma volume of graft neurons is around 75% greater than those in the contralateral host region, a relationship that is similar if intramesencephalic and intrastriatal grafts are analyzed separately (not shown). Species differences in neuronal size have been used previously to distinguish between graft and host neurons in a neuronal xenograft situation (36), and these data strongly indicate that NeuN-positive cells within the presumptive graft mass are donor derived. (b) Neuronal soma volume of NeuN-immunoreactive cells within intrastriatal (black bars) and intramesencephalic grafts (gray bars). Twelve randomly selected cells in three representative grafts in each site were measured. The frequency histogram is shown with mean values (+SEM) shown in the inset. The distribution of cell volumes does not vary between grafts in the two sites ($\chi^2_{12} = 10.67$, ns). These data suggest that pENPs have adopt similar morphological phenotypes irrespective of the location in which they are placed. (c–h) NeuN immunostaining of neurons; note the densely stained nucleus and more lightly stained soma. Several morphological phenotypes were apparent in grafts in both sites. Ovoid, immature-looking cells were frequent (c, d) some of which appeared unipolar with a single process (e). Larger, more mature-looking cells were also evident: both polygonal, multipolar cells (f) and pyramidal-shaped cells with obvious axonal hillocks (scale 10 μm).

the hemiparkinsonian rat with the aim of exploiting the nature of the xenograft paradigm to explore the potential for such cells to develop into projection neurons that may be useful for therapeutic neural circuit reconstruction.

This study has demonstrated that neural precursor cells isolated from the developing pig brain proliferate as spheres in response to EGF and FGF-2 and can be propagated using methods optimized for the growth of human neural precursor cells (65). These cells display properties analogous to those described for ENPs derived from other species in that they divide to incorporate BrdU, express nestin (an intermediate filament protein which is characteristic of, although not exclusive to, neural stem cells (40)), and differentiate into neurons, astrocytes, and oligodendrocytes on mitogen withdrawal. Such observations go further to suggest that neural stem-like cells are highly conserved through phylogeny, although some differences in behavior between species have been described (64).

Clonal studies in rodents have suggested that even after a short period of exposure to mitogens neural stem cells may constitute the majority of cells in such populations (37). However, since these multipotential cells divide asymmetrically in culture to give rise to another stem cell and a lineage-restricted progenitor (38, 50) populations of cells grown under conditions which promote the division and expansion of neural stem cells (even if originating from a single cell) are actually a mixture of precursor cells at different stages of phenotypic commitment. Thus, it seems likely that the pENPs are a mixture of stem cells and restricted progenitors, although the population-based approach used in this study to maximize the number of cells available for grafting precludes firm conclusions pertaining to nature and lineage relationships of cells within the cultures.

On xenotransplantation in the CsA-treated, 6-OHDA-lesioned rat, all grafts of pENPs survived. Examination of markers known to be correlated with the

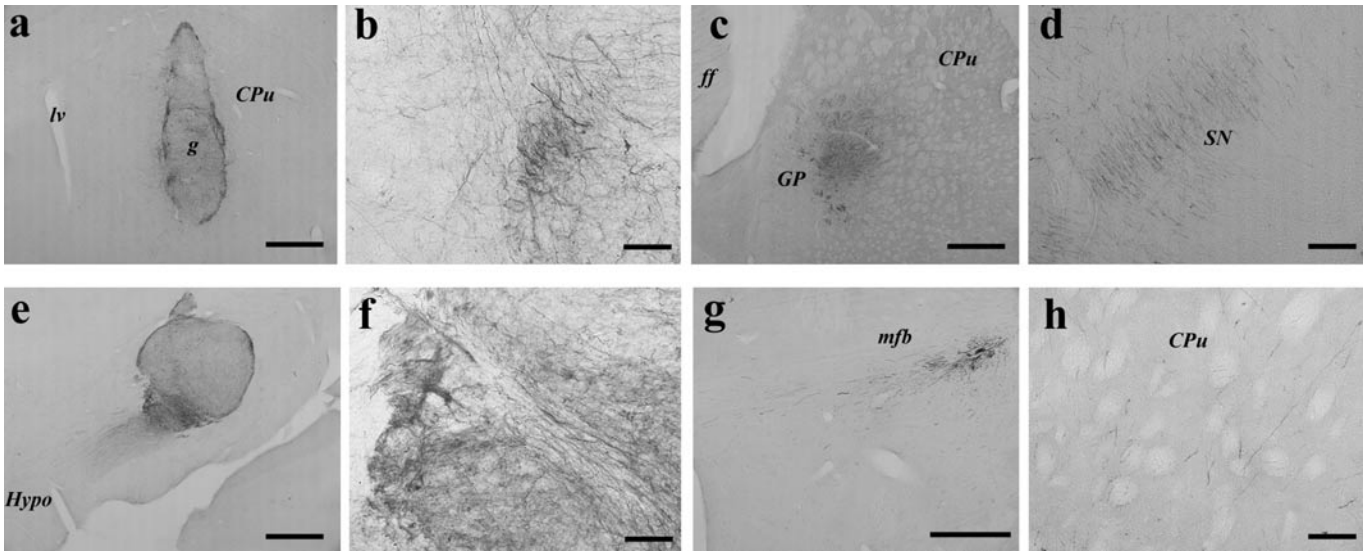

FIG. 5. Pig-specific NF_{70} immunohistochemistry. (a–d) Projections of a typical intrastriatal graft and (e–h) a typical intramesencephalic graft, the size of which meant that it encroached on the ventral tegmental area and this may contribute to the innervation of ventral striatal regions seen. (a) and (e) NF_{70} fibers are present throughout the entirety of the graft mass; (b and f) Details of (a) and (e), respectively, and large numbers of fibers streaming over the graft–host borders. Projections of pENPs placed intrastriatally included those appropriate for the site of implantation, such as (c) the pallidum and (d) the substantia nigra. Similarly intramesencephalic grafts were seen to project, via normal mesencephalic outflows such as the medial forebrain bundle (g), to appropriate sites such as the caudate-putamen (h). *lv*, lateral ventricle; *g*, graft; *CPu*, caudate-putamen; *ff*, fimbria-fornix; *GP*, globus pallidus; *SN*, substantia nigra; Hypo, hypothalamus. (Bars: a, c, d, 1000 μm; b, f, d, h 200 μm; g 500 μm).

rejection of intracerebral grafts (3, 8, 48) suggested that a rejection response had not been elicited 9 weeks postgrafting. CsA does not fully abolish the rejection of primary tissue xenografts and a proportion of primary tissue grafts would be expected to be undergoing rejection at this time point (46, 47, 49). The robust survival of pENPs in this paradigm is likely related to the reduced expression of histocompatibility epitopes by pENP cell suspensions relative to equivalent primary cell grafts (3, 33, 43).

FIG. 6. Immunohistochemistry for pig-specific synaptobrevin. This punctate staining pattern was seen in distant targets in the same distribution as the NF_{70}-positive fibers and suggest the formation of synapses by ENP-derived neurons. Arrows indicate individual synapses (scale 20 μm).

Following transplantation, the neuronal differentiation of pENPs was demonstrated by the use of a pig-specific NF_{70} antibody (see below) and by immunohistochemistry for NeuN. In the xenograft situation, where cell size clearly differs between species, quantification of neuronal size within a presumptive graft mass has been used previously to differentiate between graft and host neurons (36). The larger size of donor neurons compared to those of the host rat allowed NeuN-positive neurons within the presumptive graft mass to be identified with some certainty as donor derived and further suggested that donor–host neuronal chimerism within the graft mass was negligible. On this basis, the number of neurons that had differentiated from pENPs at 9 weeks could be determined. This revealed that, as a proportion of cells transplanted, some 50–90% had adopted a neuronal phenotype, and this did not vary depending on the implantation site. This figure is most likely a product of the dynamic processes of cell proliferation and death that probably occur prior to the sacrifice point and will be further affected by the inability to identify any donor cells that have migrated away from the graft mass (30, 62). It is possible that the proportion of cells that adopt a neuronal fate may diminish with increased expansion *in vitro* (62), although this has not been specifically addressed in this study.

A key aim of this study was to determine whether grafted pENPs had the ability to differentiate into projection neurons that may be useful for neural circuit

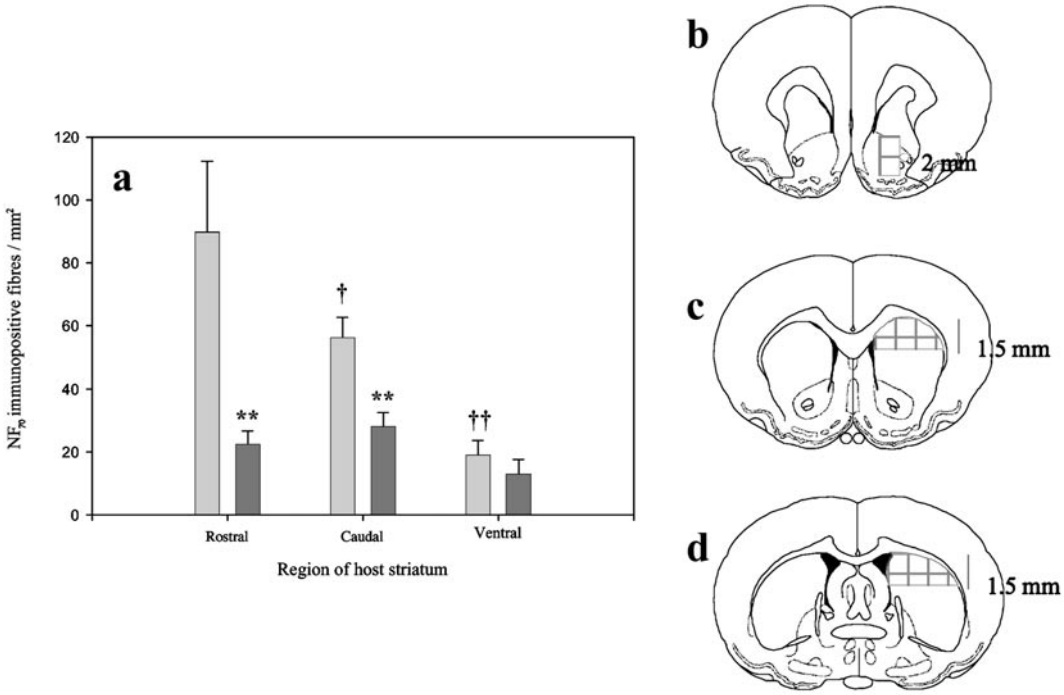

FIG. 7. (a) Quantification of graft-derived, NF_{70}-positive fibers in the host striatum. The number of fibers derived from intrastriatal grafts is significantly greater than that derived from by intramesencephalic grafts at all levels except in the ventral striatum ($*P < 0.05$, $**P < 0.01$). Intrastriatal grafts project to the rostral striatum more than the caudal/ventral regions, although this may reflect the graft placement rather than a specific tropism ($\dagger P < 0.05$, $\dagger\dagger P < 0.01$). There are no significant differences in the number of fibers in the three target sites by intramesencephalic grafts. Light grey bars refer to intrastriatal grafts and dark grey bars refer to intramesencephalic grafts. (b–d) The anatomical location of the counting frames (b) ventral; (c) dorsal, rostral; and (d) dorsal, caudal.

reconstruction. Standard rat-to-rat allografts of primary fetal neural tissue have only a limited capacity to extend axons, such that intranigral implants fail to reinnervate the striatum in adult rat models of PD (9, 11, 24). Various lines of evidence implicate inhibitory factors associated with mature white matter tracts in restricting axogenesis (2, 9, 13, 27, 78). In contrast, primary tissue xenografts derived from the developing human and pig brain show prolific axonal outgrowth in the same model (35, 76). The reasons for this difference are uncertain; one possibility is that pig (and human) neurons have a greater intrinsic growth capacity (28). An alternative is that species differences in growth inhibitory molecules and/or their receptors might make xenografted axons less responsive to inhibitory cues (69; C. B. Hurelbrink, unpublished observations). In light of this latter possibility it has been speculated that there may be a primary advantage in the use of xenogeneic (i.e., porcine) tissue as opposed to allogeneic (i.e., human) in a clinical setting to facilitate improved circuit repair. The current study has sought to exploit this phenomenon experimentally.

pENPs differentiated into neurons that had the ability to project extensively along white matter tracts; furthermore, staining with pig-specific synaptobrevin (20) suggested that pENP-derived neurons could synapse with host cells. Interestingly, pENPs were not restricted in the targets to which they could project and projections included those appropriate for their site of implantation, such that intramesencephalic grafts projected to the striatal complex and intrastriatal pENPs projected abundantly to the pallidum, entopeduncular nucleus, and the substantia nigra. This is in contrast with the situation reported for primary tissue grafts that are less plastic and are restricted by their region of origin in the range of targets they can innervate (20, 21, 32, 34, 74–76). This difference probably further reflects the immaturity and plasticity of pENP-derived neurons and may potentially make them uniquely flexible for therapeutic cell replacement where anatomical reconstruction is necessary or advantageous.

The proportion of cells that developed spontaneously on transplantation to express a presumptive dopaminergic phenotype was small and neither intranigral nor intrastriatal grafts mediated a positive functional effect. Several other studies have failed to report any functional benefit of intrastriatal (human) ENP transplants in the PD model (45, 63, 70) and we can speculate as to the reasons for this. Evidence suggests that ENPs derived from different regions of the developing brain are equivalent in terms of their ability to adopt neurochemical phenotypes and that adoption of a mid-

brain dopaminergic phenotype is rare, even when ENPs are derived from the ventral mesencephalon (VM) (12, 16, 19, 30, 54). Nonetheless, studies that have claimed (variable and incomplete) functional benefit from ENP grafts in the PD model have derived ENPs from fetal brain containing the VM (55, 59, 62). Therefore, it is possible that some phenotypic specification may remain when precursors are explanted and expanded and that the derivation of pENPs from the VM (rather than the cortical plate used in this and several other studies) may result in an increased yield of TH-positive neurons.

Furthermore, studies using primary tissue grafts have suggested that the environment of the dopamine-denervated adult striatum may be inadequate to fully direct the dopaminergic differentiation and maturation of uncommitted progenitors (57), and one may speculate that species differences might make the xenograft environment even less favorable for ENP differentiation. One possible approach to this problem is to induce ENPs differentiation prior to transplantation (59). Encouragingly, a number of specific factors and culture conditions have recently been suggested to promote stem-like cells to adopt a dopaminergic fate, although the increases in yield to date have been modest (18, 19, 39, 42, 58, 66, 71, 80).

The neurochemical phenotype of the non-TH-positive neurons developing in the pENP grafts is unknown. The morphology of neurons derived from ENPs was heterogenous and may indicate the emergence of multiple phenotypes, although this remains to be clarified. The morphological heterogeneity may also derive from neurons being at different stages of maturity. Work with human ENPs has shown that multiple neurotransmitter phenotypes may emerge, but even following protracted time *in vivo* the majority of neurons remain of unidentified phenotype (30).

It is also of note in the current study that there were no clear differences between neurons that developed in the midbrain and those that developed in the striatum. This is in apparent contrast to the situation when human or rat ENPs are grafted into the developing brain or neurogenic regions of the adult brain when neurons seem to adopt a morphological phenotype appropriate for their site of integration (15, 53, 60, 67). This difference may partly be attributable to environmental differences between actively neurogenic regions and the largely postmitotic adult substantia nigra and striatum. Furthermore, in contrast to the cited studies, neurons that were examined morphologically in the current study had developed within the local environment of a graft mass, rather than following migration into the host neuropil and exposure to the local host environment.

Vimentin is an intermediate filament expressed in radial glia and immature astrocytes early in development (17, 56) and has been used as a marker for astroglial precursors emerging from stem-like cells *in vivo* (22). Vimentin-positive cells with an astrocytic morphology were apparent throughout the pENP graft mass, and it seems likely given the generation of astroglia from pENPs *in vitro* that these vimentin-positive cells represent pENP-derived astrocytes. The absence of a pig-specific glial marker, however, precludes their definitive identification as graft-derived, and it cannot be excluded that such cells constitute reactive host astrocytes that have migrated into the graft mass (7, 17).

In conclusion, exploiting the experimental advantages of xenografts has allowed us to demonstrate that populations of expanded neural precursor cells have the ability to develop into neurons that can project through white matter tracts and apparently can synapse with host cells. This study has implications for the development of novel cellular therapies in neurodegenerative diseases where circuit reconstruction would be advantageous.

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