Published in final edited form as:

Cell Transplant. 2004 ; 13(5): 535–547.

Studies on the Differentiation of Dopaminergic Traits in Human Neural Progenitor Cells In Vitro and In Vivo

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Abstract

The development of cell replacement therapies for the treatment of neurodegenerative disorders such as Parkinson's disease (PD) may depend upon the successful differentiation of human neural stem/progenitor cells into dopamine (DA) neurons. We show here that primary human neural progenitors (HNPs) can be expanded and maintained in culture both as neurospheres (NSPs) and attached monolayers where they develop into neurons and glia. When transplanted into the 6-hydroxydopamine-lesioned rat striatum, undifferentiated NSPs survive longer (60% graft survival at 8–16 weeks vs. 30% graft survival at 8–13 weeks) and migrate farther than their attached counterparts. While both NSP and attached cells continue to express neuronal traits after transplantation, the spontaneous expression of differentiated transmitter-related traits is not observed in either cell type. However, following predifferentiation in culture using a previously described cocktail of reagents, approximately 25% of HNPs can permanently express the DA enzyme tyrosine hydroxylase (TH), even following replating and removal of the DA differentiation cocktail. When these predifferentiated HNPs are transplanted into the brain, however, TH staining is not observed, either because expression is lost or TH-expressing cells preferentially die. Consistent with the latter view is a decrease in total cell survival and migration, and an enhanced glial response in these grafts. In contrast, we found that the overall survival of HNPs is improved when cells engraft near blood vessels or CSF compartments or when they are placed into an intact unlesioned brain, suggesting that there are factors, as yet unidentified, that can better support the development of engrafted HNPs.

Keywords

Neural stem cells; Progenitor cells; Dopamine; Differentiation; Tyrosine hydroxylase; Transplantation; Parkinson's disease; 6-Hydroxydopamine lesion

INTRODUCTION

Despite decades of intense investigative efforts, there remains no reliable way to either prevent or rescue dopa-mine (DA) neurons from the progressive degeneration that occurs during aging or in Parkinson's disease (PD). Because clinical diagnosis almost always occurs after the vast majority of DA neurons have already been destroyed, the development of ways in which to replace lost tissue with transplanted cells capable of DAergic function has become a prime focus of research (3,5,19,21–23,38,42,55). However, these strategies depend for their success

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on the identification of a reliable source of transplantable DA neurons and factors relevant to their growth and survival.

Although transplants of fetal human DA neurons in PD patients have met with considerable success (11,20,21,24,25,42-44,49,54,58,71), there are many practical (limited supply, cell heterogeneity, variable procurement methods, etc.) and ethical issues that limit the utility of these cells. In an effort to overcome these obstacles, recent studies have focused on the promising possibility that DA neurons can instead be generated from multi-potential stem or precursor cells (2,6,39,52,62,63,67,70). Because these cells can self-renew, they potentially provide an abundant supply of transplantable tissue. However, they also pose their own unique challenge; unlike fetal neurons, stem/progenitor cells must first be differentiated to express the appropriate DA phenotypic traits, either in culture or in the brain itself.

Indeed, our previous studies have demonstrated that it is possible to coax certain embryonic cells to express DA traits in culture (14,29-31,60,61) by incubating them with a differentiation cocktail containing fibroblast growth factor 1 (FGF1) and a number of cofactors, including activators of the protein kinase A and C pathways (14-18). Likewise, a number of other laboratories have also demonstrated various degrees of success directing the DA differentiation of stem (37,40), progenitor cells (9,12,45,48,53,56,59,62), or their conditionally immortalized counterparts (4,57,75,76).

Similarly, it is possible to differentiate a DA pheno-type in a fraction of cells once they are transplanted in vivo. Thus, when murine or rodent embryonic stem (ES) cells (7,13,36,37) or more committed neural progenitor cells (48) are transplanted into the parkinsonian rat brain, a portion of the cells express DA phenotypic traits. Indeed, under certain conditions, our laboratory further found that mouse neural progenitor cells can survive and migrate in the brain and that virtually all cells will spontaneously differentiate into neurons that express the DA enzymes tyrosine hydroxylase (TH) and aromatic L-amino decarboxylase (AADC) (73,74) even in the absence of genetic or environmental manipulation. Thus far, however, in studies using human neural progenitor cells, TH expression has only rarely (41), and in some cases only transiently (51), been observed in engrafted cells, and with no functional consequence. The goal of the present study was to determine whether, under different growth conditions in vitro, human neural progenitor cells might be more easily persuaded to express TH in the transplant. To do so, human neural progenitors (HNPs) were expanded in culture either as neurospheres (NSP) or as a monolayer of attached cells and transplanted into 6-hydroxydopamine (6-OHDA)-lesioned rats in the undifferentiated state or following their predifferentiation into TH-expressing cells in culture.

MATERIALS AND METHODS

Cell Culture

Primary HNPs (derived from 19–21-week fetuses) grown as NSPs were generously supplied by ScienCell Research Laboratories (San Diego, CA.); those grown as attached cells were purchased from Clonexpress, Inc. (Gaithersburg, MD.) HNPs were maintained in culture according to suppliers' directions on either neural stem cell media (ScienCell) or media containing NSC supplement (Clonexpress).

Cells were grown on noncoated Corning T75 tissue culture flasks until harvest (Versene; 3–5 min, RT). They were then spun at low speed (<1000 rpm) and re-suspended in Hank's BSS with added glucose (6 mg/ml) and glutamine (204 mg/ml) at a final concentration of 2.5×10^5 cells/5 ml for transplantation. Adherent cells were plated onto polyornithine-coated culture dishes, maintained for 1 day in culture.

Differentiation Protocol

NSPs were plated on the day of arrival from the supplier and, in some cases, immediately incubated overnight in the DA differentiation cocktail (defined serum-free medium containing 10 ng/ml FGF1, 200 nM TPA, 10 μ M DA, 65 μ M IBMX, and 15 μ M forskolin). The next day cultures were rinsed, harvested, and transplanted into parkinsonian rats. Adherent HNP were plated onto polyornithine-coated slides for 1–3 days, incubated overnight with the DA cocktail, and then gently harvested (0.05% trypsin) for transplant. Some NSPs and attached HNPs in the cell suspension prepared for transplantation were replated onto polyornithine-coated slides, fixed the following day, and stained to verify and quantify the induction of TH.

6-OHDA Lesions

As described previously (32), rats were anesthetized with sodium pentobarbital (30 mg/kg, IP) and placed in a stereotaxic apparatus (Kopf Instruments). A 26-gauge Hamilton syringe containing 6-OHDA (Sigma; 20 μ g/ml in 4 μ l saline containing 0.2 mg/ml ascorbate) was lowered into the right medial forebrain bundle (AP: -4.4 mm, ML: -1.2 mm, DV: -7.8 mm from bregma). The 6-OHDA solution was gradually injected at a rate of 1 μ l/min. Three weeks later, all lesions were verified by assessment of rotational behavior in an automated roto-meter system (Columbus Instruments) following amphetamine injection (5 mg/kg, IP).

Transplantation

Animals ($N = 66$; 4 animals died) with verifiable 6-OHDA lesions (>5 ipsilateral turns/min) were implanted with 5 μ l of HNPs (2.5×10^5 cells/5 μ l) in the striatum on the side ipsilateral to the lesion using procedures described previously (73,74). Cells (2.5 μ l) were stereotactically injected at four depths (AP: +1.2 mm, ML: -2.7 mm, DV: -5.4, -4.9, -4.4, -3.9 mm). In other experiments, cells were similarly grafted into either the intact ($N = 35$; 2 animals died) or unlesioned ($N = 3$) striatum contralateral to the 6-OHDA lesion (2.5 μ l of cells at each level; AP: +1.2 mm, ML: +2.7 mm, DV: -5.4 and -4.9 mm). All transplant recipients received cyclosporine A (10–15 mg/kg, IP) five times per week, beginning on the day before transplantation. Animals were sacrificed at various times following transplantation, ranging from 1 day to 20 weeks.

Immunocytochemistry

Rats were perfused with 500 ml of cold (4°C) periodate-lysine-paraformaldehyde (4% PLP) and sections were cut at 30 μ m on a freezing microtome. Cell cultures were similarly fixed overnight and processed for single or double label immunocytochemistry using immunofluorescence (IF) or immunoperoxidase (IP). Fixed cultures or brain sections were processed with monoclonal antibodies to human nuclear protein (Hunu; 1:25), β -tubulin III (β -tub III; 1:800), glial fibrillary acidic protein (GFAP; 1:800) or polyclonal antibodies to nestin (Nstn; 1:200), neurofilament (NF; 1:1000), neuronal specific enolase (NSE; 1:500), TH (1:500), serotonin (5-HT; 1:8000), or choline acetyltransferase (ChAT; 1:2500). All antibodies were purchased from Chemicon except for β -tub III, which was kindly provided by Dr. J. Provencio (Philadelphia, PA). For immunofluorescence, the following secondary antibodies were purchased from Jackson Immunoresearch: donkey anti-rbFITC (1:50), donkey anti-mouse-FITC (1:50), donkey anti-rabbit-rhodamine (1:150), donkey anti-mouse-rhodamine (1:200). IP staining was developed using the ABC method (Vector Labs) as described previously (18). All micrographs were analyzed on a Nikon-Scanalytics Image System.

RESULTS

Studies of HNPs in Culture

As can be seen in Figure 1, undifferentiated HNPs thrive in culture either as NSPs (Fig. 1A) or as attached monolayers (Fig. 1B). However, undifferentiated NSPs, which remain as a sphere of rounded cells, appear more undeveloped than attached HNPs, which extend long and elaborate neuritic processes. Consistent with their presumed undeveloped state, NSPs can be readily passaged at least 10 times in culture while attached HNPs continue to divide and expand in culture only up to three passages.

When cultures of undifferentiated HNPs were examined immunocytochemically for the presence of cell-specific markers (Fig. 2), HNPs expressed low levels of the neuronal markers β -tub III (Fig. 2A), neurofilament (Fig. 2B), and NSE (Fig. 2C) and the glial marker protein GFAP (Fig. 2D), but did not express, except on rare occasion, more differentiated phenotypic traits like TH (Fig. 2E, F) or other neurotransmitter phenotypic markers, like 5-HT or ChAT (data not shown).

In contrast, when HNPs were grown in culture 1–4 days and then incubated overnight in a differentiation cocktail (10 ng/ml FGF1, 200 nM TPA, 10 μ M DA, 65 μ M IBMX, and 15 μ M forskolin) (Fig. 3), which can induce the novel expression of TH in other stem/progenitor cells (14,29,30,60,61), predifferentiated HNPs developed into both neurons that darkly stained for the neuronal markers β -tub III (Fig. 3A), neurofilament (Fig. 3B), and NSE (Fig. 3C) and glia that stained for GFAP (Fig. 3D). Moreover, approximately 25% of these predifferentiated cells stained intensely for TH, both in attached cell (Fig. 3E) and NSP (Fig. 3F) cultures. Importantly, predifferentiated HNPs continued to express TH even 7 days after the removal of the DA differentiation cocktail, suggesting that once expression of the TH gene has been “turned on,” it remained on indefinitely in differentiated HNPs.

Studies of HNPs Following Transplantation

We next compared the survival, migration, and differentiation of undifferentiated HNPs after their transplantation into the parkinsonian (6-OHDA-lesioned) rat striatum. Shown in Figure 4 is a time course of the survival (Fig. 4A-H) and migration (Fig. 4I-L) of undifferentiated HNPs after transplantation into the striatum of a 6-OHDA-lesioned rat. HNPs were derived from either NSP cultures (Fig. 4A-D, I, J) or attached monolayers (Fig. 4E-H, K, L). In order to distinguish the engrafted HNPs from adult host rat tissue, brain sections were double labeled for the human nuclear marker, Hunu (red), and progenitor marker, nestin (green). While both NSPs and attached HNPs survived and migrated well in the brain, NSPs endured longer (60% graft survival at 8–16 weeks) than cells maintained as a monolayer prior to transplantation (30% graft survival at 8–13 weeks). While both NSPs and attached HNPs migrated as single cells, oftentimes following white fiber tracks to become widely dispersed in the host striatum (Fig. 4I-L) and cortex, only NSPs were found to have crossed the mid-line to implant in the contralateral cerebral cortex. Interestingly, in the oldest grafts (20 weeks), transplanted cells were selectively located in the region of the subventricular zone, an area where host embryonic and adult stem cells are thought to reside (26,46,47,64,65,68,69).

Of critical importance was the fact that no TH expression was observed in transplanted undifferentiated NSPs or attached HNPs at any of the time points examined (data not shown). Thus, unlike previous studies using clonal mouse neural progenitors (73,74), primary human progenitors did not spontaneously express TH, even after 20 weeks in vivo.

Consequently, experiments were initiated to predifferentiate HNPs in culture to express DA traits prior to their transplantation in vivo. When the time course of survival of predifferentiated HNPs (Fig. 5; nestin = green; Hunu = red) was examined and compared with undifferentiated

HNPs, we found that the overall length of time cells survived in the graft decreased in more developed cells. Thus, while undifferentiated NSPs survived for months posttransplantation, predifferentiated NSPs were present in much lower numbers at comparable times (e.g., compare Fig. 4C with Fig. 5D). In fact, by 8 weeks posttransplantation, few NSPs (Fig. 5D) and almost no attached cells (Fig. 5H) remained in the graft. Moreover, there was little migration of HNPs away from the graft site, unlike their undifferentiated counterparts. Instead, predifferentiated HNPs behaved similarly to older fetal cells, permanently settling almost exclusively at the graft site. Of further significance, although a significant number (~25%) of NSPs or attached HNPs expressed TH after predifferentiation in culture, no TH-expressing HNPs were observed in the graft at any of the time points examined (1 day to 8 weeks posttransplantation) in either the intact or 6-OHDA-lesioned brain. Nor did cells express traits of other neurotransmitter systems like 5-HT or ChAT (data not shown).

Associated with the reduced survival and migration of predifferentiated HNPs was a concomitant host glial response (Fig. 6). Thus, undifferentiated NSP cells (Hunu or nestin: green) produced little glial reaction (GFAP: red) in the region of the graft at 2 weeks posttransplantation (Fig. 6A), while attached cells (Fig. 6B) produced a mild gliosis. However, this response was greatly exaggerated when HNPs were first predifferentiated (Fig. 6C, D) in culture prior to transplantation. Despite a marked host glial reaction, there was little evidence for glial differentiation of HNPs (yellow colabeled GFAP+/Hunu+ HNPs) except at the edge of the graft proper (see Fig. 6B, inset). Although many HNPs can express GFAP (Fig. 2D and Fig. 3D) in vitro, it appears that the vast majority of HNPs in vivo follow a neuronal differentiative pathway (Fig. 8).

Interestingly, regardless of their differentiation status, HNPs appeared healthier and more highly developed, with a larger nucleus and more extensive processes (Fig. 7) when the placement of the transplant caused cells to contact CSF in the subarachnoid space (Fig. 7A, D, G, J) or lateral ventricle (Fig. 7B, C, E, F), or when cells were adjacent to a blood vessel (Fig. 7H, I, K, L). In some instances, cells matured such that nestin was no longer expressed (red only cells in Fig. 7G). Although TH-expressing cells were not observed at these locations, the importance of graft placement for overall HNP development was especially evident when the neuronal marker β -tub III was examined (Fig. 8; nestin: green; Hunu: red; β -tub III: brown immunoperoxidase label). Regardless of whether cells were predifferentiated (Fig. 8A-C) or left undifferentiated (Fig. 8D-I), neuronal development generally appeared to be more advanced in cells found close to the needle entry site in the cortex (i.e., when cells were in close proximity to CSF) (Fig. 8A-F) and less evident in those cells that remained confined to the body of the graft, particularly those that were originally derived from NSPs (Fig. 8G-I).

Lastly, to test whether a DA-depleting lesion by 6-OHDA was itself impacting the development of en-grafted HNPs, cells were transplanted into the striatum of an intact rat or into the unlesioned striatum on the side contralateral to a 6-OHDA lesion of a rat (Fig. 9). We consistently found that HNPs, even those harvested from the same flask, survived in greater numbers and for a longer time when transplanted to the intact (Fig. 9A) or contralateral unlesioned (Fig. 9C) striatum compared with the 6-OHDA-lesioned striatum (Fig. 9B, D), suggesting that the lesioned brain contained intrinsic factors that were deleterious to HNP survival. Moreover, these effects were further exacerbated with increased injury to the brain. Thus, HNPs survived less well in animals with bilateral grafts (on both the contralateral and ipsilateral sides) compared with animals with single grafts (e.g., compare Fig. 9D with B). Importantly, however, the overall improvement in HNP survival in the intact striatum did not lead to the further differentiation of a DA phenotype in these cells (no TH+ cells observed; data not shown).

DISCUSSION

Whereas earlier studies have examined primary human NSPs in culture (9,56,59) and following their transplantation in vivo (1,8,41,50,51), this is the first study to compare the survival, growth, and differentiation of NSP-derived progenitors with that of human HNPs generated from attached monolayers and to follow their fate in vivo after their predifferentiation in culture to express DA traits.

We found that, even in the absence of added differentiation cues, both NSPs and attached HNPs developed into neurons and glia in culture, staining for specific protein markers of these cell types. Although attached cells appeared morphologically and biochemically more developed than NSPs, neither cell type differentiated further in vitro to spontaneously express neurotransmitter-associated traits, such as the DA enzyme TH.

After transplantation of undifferentiated NSPs and attached HNPs in vivo, we found that both cell types survived for extended periods and traveled long distances via white fiber tracts to populate the host striatum and cerebral cortex. However, NSPs were more long lived (20 vs. 13 weeks) and migrated farther (even to the opposite side of the brain) than once-attached cells, suggesting that HNPs fared better in vivo if they had been maintained beforehand in a less developed state in vitro. Even though HNPs gave rise to cells that stained for both neuronal and glial markers in vitro, following transplantation, HNPs were predominantly β -tub III+ with relatively few cells staining for the glial marker GFAP. Thus, in vivo, cues appeared to preferentially promote the neuronal development of undifferentiated HNPs and/or the survival of predifferentiated neurons over glia. Despite this fact, we found little evidence for the further differentiation of neurotransmitter traits in these nascent neurons. Thus, engrafted HNPs did not express TH or other neurotransmitters. This is consistent with previous reports of human NSPs, where the unprompted expression of TH was only rarely (41) or transiently (51) seen in engrafted HNPs. These findings, however, markedly contrast with our earlier studies using mouse neural progenitors, which demonstrated that essentially all cells could spontaneously express TH following transplantation into the intact or 6-OHDA lesioned brain (73,74).

In examining the disparity between these models, we found a number of potentially important differences in addition to species. First, mouse progenitor cells were derived from a line originally isolated from the newborn cerebellum, a structure that contains both mature and immature cells, while primary human cells were predominantly derived from the cerebral cortex of midgestational fetuses (ranging in age from 19 to 21 weeks). These differences in brain region and embryonic age could greatly influence developmental potentiality. Indeed, it has been postulated that the brain region from which progenitors are derived may prescribe their range of possible fates. In fact, studies in culture indicate that, because of lineage restrictions, human cortical progenitors may not be capable of TH expression (59). Consequently, it may be necessary to isolate human progenitors from other, more plastic, regions in the brain. Also, of significance in the differentiation of DA traits in progenitors is the age of the source tissue. Developmental changes in membrane receptors, signaling molecules, transcription factors, etc., that occur over time could greatly affect the ability of cells to read and interpret DA differentiation cues. One indication of that fact is that only progenitors derived from high confluence/high passage cultures in our mouse studies differentiated along neuronal lines and expressed TH in vivo (74). Similarly, only HNPs first treated with an in vitro "priming" protocol that differentiated them into nearly pure neurons were able to express cholinergic traits after engraftment (72). These findings raise the hope that it may be possible to define conditions in culture that similarly "prime" human progenitors, making them more competent to spontaneously express TH in vivo.

Regardless of lineage restrictions, we and others have found that, in culture, it is indeed possible to overcome such obstacles to expressing TH, at least in a fraction of cells (9,12,37,40,45,48, 53,56,62,75,76). Thus, in this study, after overnight incubation in culture with our previously described DA differentiation cocktail (16,17), nearly a quarter of human HNPs were persuaded to acquire DA phenotypic traits. It is noteworthy that both HNPs and their immortalized counterparts, hNTs (29,30) not only expressed the enzyme but continued to do so even after removal of the differentiation factors, presumably into perpetuity. In contrast, mouse progenitor cells can only transiently express TH and only when directly in contact with the factors (14-18,31,60,61), suggesting that the TH enzyme may be subject to different regulatory controls in mouse and human. Indeed, in our preliminary gel shift studies, the nuclear proteins binding several important TH regulatory sites, such as the AP1 site, differed when TH was expressed in mouse (27) versus human progenitor cells (33). Because most of our understanding of TH regulation derives from studies of the rat gene, subtle but important differences may prove critical as we attempt to express the enzyme in human stem cells.

Importantly, when those HNPs that were predifferentiated to express TH in culture were subsequently transplanted into the 6-OHDA-treated rat striatum, we found no indication of TH in the graft, even at the earliest time examined (1 day), signifying a questionable fate *in vivo*. It is not clear whether this owes to the loss of TH expression by these cells or the selective death of this particular HNP population in the graft. However, given the fact that, in culture, TH was not downregulated over long periods of time, it seems highly unlikely that expression would be lost after only 1 day *in vivo*. Instead, a number of facts suggest that these highly differentiated cells may have undergone a premature death *in vivo*. First, attached TH-expressing HNPs elicited long and complex processes in culture, even when grown only 1–2 days prior to their transplantation. Upon harvest, processes may have been so severely disrupted that cells were unable to recover after transplantation. However, several facts suggest that this reason alone cannot explain our results, including that: 1) predifferentiated TH+HNPs can be replated in culture (a process that causes neurite loss) where they thrive and continue to express TH for days; 2) many non-TH-expressing HNPs elicit long processes in culture but still survive harvest and transplantation; and 3) TH+ predifferentiated NSPs (which do not elaborate long processes and do not require disruption from an adherent substrate) do not survive transplantation. Thus, other differences must also contribute to the loss of TH cells *in vivo*. One possibility is that predifferentiation exposes HNPs to reagents (IBMX, forskolin, TPA) that can be cytotoxic. Although the brief overnight exposure used in the differentiation protocol does not affect cell viability *in vitro*, it may increase the vulnerability of these cells to potentially harmful injury-related factors found at the transplant site, including immune/inflammatory cytokines released by neutrophils, macrophages, microglia, and astrocytes (10,34,35,45). Consistent with this view is the marked reactivity of host astroglial cells observed surrounding the grafts of predifferentiated HNPs in this study. According to this scenario, HNPs possibly damaged by the predifferentiation process and by their harvest from culture cause a deleterious host response after transplantation *in vivo*. This, combined with other trauma-related elements (cells/factors) generated during the transplantation process, may ultimately contribute to the early demise of HNPs.

Somewhat paradoxically, other elements present in the graft environment appear to improve the overall survival and well-being of HNPs. Thus, we found that both undifferentiated and predifferentiated HNPs that contacted CSF compartments, or those that resided in close proximity to blood vessels, lived longer and appeared healthier, with a more robust neuritic network, than those embedded in the parenchyma. Likewise, HNPs transplanted into an unlesioned striatum fared better than those in the 6-OHDA-treated rat, suggesting that the intact brain also provided a more hospitable environment for HNPs. Although none of these conditions enhanced the survival of TH-expressing HNPs, taken together, these findings

suggest that there are factors, as yet unidentified in CSF, blood, and/or the intact brain, that can better support the general development of engrafted HNP.

Although we have not yet discovered the exact conditions needed to either spontaneously induce TH expression in undifferentiated HNP or enhance the survival of more mature predifferentiated cells in the graft, the present study raises a number of promising directions worthy of our pursuit. Perhaps by obtaining HNP from age and/or tissue-appropriate sources, we can better promote the constitutive expression of TH in undifferentiated HNP. Or possibly HNP in vivo, as those in vitro, can be coaxed into expressing TH by using or mimicking the effects of the DA differentiation cocktail in the brain. Alternatively, it may be possible to prevent the death of predifferentiated TH-expressing HNP by improving the ways in which process-bearing cells are harvested from culture before transplantation or by devising interventions that can block the actions of harmful factors present after transplantation. Conversely, perhaps trophic agents can be identified that better support the survival of TH-expressing progenitors in the graft. Future studies will be needed to fully address these issues as we move forward in the development of cell replacement therapies for Parkinson's disease.

ACKNOWLEDGMENTS

We thank Ms. Yubao Jiang for the preparation of immunohistochemical materials and for assistance preparing 6-OHDA-lesioned rats, and Dr. Javier Proven-cio (Philadelphia, PA) for the kind gift of β -tubulin III antibodies. This work was supported by NIH NS24204, NS32519 and NS 43309 (to L.I.) and a Parkinson's Disease Foundation award (to M.Y.).

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Figure 1.

Phase contrast photomicrograph of HNPs grown for several days in culture flasks either as neurospheres (A) or as an attached monolayer of cells (B). Note the extensive development of individual cells and their processes in (B). Calibration bar = 100 μ m in (A and B).

Figure 2. Immunocytochemical localization of β -tub III (A), NF (B), NSE (C), GFAP (D), and TH (E, F) in undifferentiated HNP cultures grown and maintained either as attached cells (A–E) or as NSPs (F). Calibration bar = 200 μ m in (A–F).

Figure 3.

Immunocytochemical localization of β -tub III (A), NF (B), NSE (C), GFAP (D), and TH (E, F) in cells of predifferentiated HNP cultures grown 3 days in culture and maintained either as attached monolayers (A–E) or as NSPs (F). Predifferentiation was achieved by overnight incubation in defined media containing 10 ng/ml FGF1, 200 nM TPA, 10 μ M DA, 65 μ M IBMX, and 15 μ M forskolin. Note the presence of dark TH staining in a fraction of differentiated HNPs. Calibration bar = 200 μ m in (A–F).

Figure 4.

Immunocytochemical localization of nestin (green) and Hunu (red) in undifferentiated HNPs following transplantation into the striata of animals with prior 6-OHDA lesions. Merged images (A–L) are shown representing the colocalization of antigens in cells grown either as NSPs (A–D, I, J) or as attached monolayers (E–H, K, L) in culture and then transplanted for 2 (A, E), 4 (B, F), 6 (C, G), or 8 (D, H) weeks in vivo. Note the presence of labeled cells in both the graft proper (A–H) and following migration to widely dispersed regions of the brain (I–L). Inset in (D) shows at higher power HNPs colabeled for Hunu and nestin in the graft. Calibration bar = 100 μ m in (A–J, L) and 200 μ m in (K).

Figure 5.

Immunocytochemical localization of nestin (green) and Hunu (red) in predifferentiated HNPs following transplantation into the striata of animals with prior 6-OHDA lesions. Merged images (A–H) are shown representing the colocalization of antigens in cells grown either as NSPs (A–D) or as attached monolayers (E–H) in culture and then transplanted for 2 (A, E), 4 (B, F), 6 (C, G), or 8 (D, H) weeks in vivo. Note the absence of labeled cells in older grafts (D, H), particularly in those that had been grown as attached cells in culture prior to transplantation (H). Calibration bar = 100 μ m in (A–H).

Figure 6.

Immunocytochemical localization of nestin or Hunu (green) and GFAP (red) in undifferentiated (A, B) or predifferentiated (C, D) HNPs after transplantation into 6-OHDA-lesioned rats. Merged images are shown representing the colocalization of antigens in cells grown either as NSPs (A) or attached cells (B–D) in culture and then transplanted for 2 (A–C) or 4 (D) weeks in vivo. Note also the presence of progenitor cells which colabeled with GFAP (yellow). Inset in (B) shows double-labeled Hunu and GFAP staining in some HNPs at the edge of the graft. Calibration bar = 100µm in (A–D).

Figure 7.

Immunocytochemical localization of nestin (green) and Hunu (red) of HNPs grown as NSPs and then transplanted into the striata of animals with prior 6-OHDA lesions. Merged images are shown representing the dual localization of antigens (A–C, G–I). Note the complex development of cells located near the subarachnoid space (Subarach: A, D, G, J), lateral ventricle (LV: B, C, E, F), or blood vessels (Bld Ves: H, I, K, L), some of which express Hunu but do not any longer express nestin [red-only cells in (G)]. Note also the presence of cells that express nestin but not Hunu [green-only cells in (G)], which may represent reactive host glia or transplanted HNPs whose Hunu+ red nucleus was not present in the section. Calibration bar = 100µm in (A, C, D, F, G, I, J, L) and 200µm in (B, E, H, K).

Figure 8. Immunocytochemical localization of nestin (green) and Hunu (red) in merged images (A, D, G) and the neuronal marker, β -tub III, in low (10 $\times$: B, E, H) and high power (40 $\times$: C, F, I) photomicrographs of transplants of predifferentiated (A–C) or undifferentiated (D–I) HNPs derived originally from NSP cultures. Note the complexity of neuronal process formation in cells close to the CSF compartment (A–F) compared with those in the graft proper (G–I). Calibration bar = 100 μ m in (A, B, D, E, G, H) and 200 μ m in (C, F, I).

Figure 9. Immunocytochemical localization of nestin (green) and Hunu (red) in merged images of HNPs derived from NSP cultures and transplanted for a minimum of 4 weeks into the intact rat striatum (A), into the 6-OHDA-lesioned striatum of a unilaterally engrafted rat (B), into the striatum contralateral (C) or ipsilateral (D) to a 6-OHDA lesion of a bilaterally engrafted rat. Calibration bar = 100 μ m in (A–D).