HUMAN NEURAL STEM CELLS MIGRATE ALONG THE NIGROSTRIATAL PATHWAY IN A PRIMATE MODEL OF PARKINSON'S DISEASE

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Abstract

Although evidence of damage-directed neural stem cell (NSC) migration has been well-documented in the rodent, to our knowledge it has never been confirmed or quantified using human NSC (hNSC) in an adult non-human primate modeling a human neurodegenerative disease state. In this report, we attempt to provide that confirmation, potentially advancing basic stem cell concepts toward clinical relevance. hNSC were implanted into the caudate nucleus (bilaterally) and substantia nigra (unilaterally) of 7, adult St. Kitts African green monkeys (*Chlorocebus sabaeus*) with previous exposure to systemic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin that disrupts the dopaminergic nigrostriatal pathway. A detailed quantitative analysis of hNSC migration patterns at two time points (4 and 7 months) following transplantation was performed. Density contour mapping of hNSCs along the dorsal-ventral and medial-lateral axes of the brain suggested that >80% of hNSCs migrated from the point of implantation to and along the impaired nigrostriatal pathway. Although 2/3 of hNSC were transplanted within the caudate, <1% of 3×10^6 total injected donor cells were identified at this site. The migrating hNSC did not appear to be pursuing a neuronal lineage. In the striatum and nigrostriatal pathway, but not in the substantia nigra, some hNSC were found to have taken a glial lineage. The property of neural stem cells to allign themselves along a neural pathway rendered dysfunctional by a given disease is potentially a valuable clinical tool.

Introduction

The directed physiologically-relevant migration of neural stem cells (NSCs) distinguishes them from other therapeutic modalities (Park et al, 1999). In models of intracerebral hemorrhage

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and ischemia, NSCs migrate to the area of infarction (An et al., 2004; Chu et al., 2003; Hayashi et al., 2006; Ishibashi et al., 2004; Jeong et al., 2003; Kelly et al., 2004; Wennersten et al., 2004; Imitola et al, 2004; Park et al, 1999, 2006). In models of multiple sclerosis or the leukodystrophies, NSCs migrate to sites of demyelination (Brundin et al., 2003; Pluchino et al, 2003; Taylor et al, 2006; Yandava et al, 1999). NSCs implanted even contralateral to brain tumors, will cross through the midline to surround the tumor (Aboody et al., 2000; Tang et al., 2003; Zhang et al., 2004). In contrast, when NSCs are implanted directly into damaged areas, they tend to remain within the injury "niche" (Aboody et al., 2000; Bosch et al., 2004; McBride et al., 2004; Park et al., 2006). In a model of Huntington's disease, the majority of NSCs implanted into the quinolinic-lesioned striatum remained within that site even after 8 weeks with none migrating to the undamaged contralateral side, suggesting that NSC migration is not a random event and is likely a directed property of NSCs (Bosch et al., 2004; McBride et al., 2004). Indeed, when NSCs are implanted into the normal adult brain (and *not* into an endogenous migratory pathway such as the rostral migratory stream), their movement away from the implant site is minimal (Englund et al., 2002; Jeong et al., 2003; Kelly et al., 2004; Ourednik et al., 2002; Zhou et al., 2003).

The phenomenon of NSCs migration to pathological sites over long distances (even from locations remote from the damage) has been explored almost exclusively in rodents with none, to our knowledge, in primates. In rodent models of PD, most studies to date have focused on cells implanted directly into the striatum, recapitulating earlier studies using dopaminergic fetal tissue transplant techniques. They report mainly on NSC which remained in the striatum (the site of lost dopaminergic input) (Burnstein et al., 2004; Wu et al., 2006; Ourednik et al., 2002; Zhou et al., 2003). When migration from the implant site was noted, the authors described NSC that migrated occasionally a short distance from the striatum via the corpus callosum, but a detailed description of their destination was not reported (Dziewczapolski et al., 2004; Liker et al., 2003; Wang et al., 2004). NSCs engineered to over express glial derived neurotrophic factor (GDNF) or nurturin also did not substantially migrate beyond the striatum but nevertheless improved motor function through retrograde transport of these trophic factors to the substantia nigra (SN) (Liu et al., 2006; Behrstock et al., 2006). So, unlike studies of other neurodegenerative disorders (i.e. ischemia, multiple sclerosis), a detailed description of NSC migration in models of PD is lacking.

Building on prior rodent studies, we implanted human NSCs (hNSCs) into MPTP-lesioned, dopamine-depleted, non-human primates. We previously detailed the phenotypical fate of those donor cells, their impact upon host dopaminergic neurons, and functional benefits (Bjugstad et al., 2005; Redmond et al., 2007). In some monkeys, the fate of transplanted hNSCs after >8 months in vivo was analyzed (Redmond et al., 2007). We found widespread migration of hNSCs throughout the brain, particularly to regions impaired directly or indirectly by MPTP with neuronal differentiation of a small proportion of NSCs appropriate to the site of migration (Redmond et al., 2007). In another set of MPTP-treated monkeys implanted with fewer hNSC and shorter survival times (4 months or 7 months) we found no neuronal differentiation, but there were reversals of MPTP-induced changes in the caudate and putamen and a specific NSC migration pattern (Bjugstad et al., 2005). Hypothesizing that NSC migration in this model is not a random event, but rather a strategic "self-positioning", we pursued a detailed quantitative analysis of hNSC migration patterns in the MPTP-lesioned monkey model of PD from the two shorter time points following transplantation. This analysis provided us with an opportunity to learn more about the hNSC migratory process in general. In so doing, we hoped to help advance stem cell concepts toward clinical relevance by providing the needed confirmation that the damage-directed NSC migration documented in the rodent does, indeed, apply to human neural stem cells in an adult non-human primate with neuropathology similar to Parkinson's disease in patients.

Methods

Cell Isolation and Preparation

Human neural stem cells (hNSC) were used for transplantation into MPTP-exposed monkeys. hNSC were isolated, expanded, cultured, and prepared for transplantation as previously described (Flax et al., 1998; Lee et al, 2007; Redmond et al, 2007). Briefly, a primary dissociated neural cell suspension was cultured from the periventricular region of the telencephalon from a 13 week human fetal cadaver. Cells were grown initially in serum and then were switched to serum-free conditions containing basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). These growth factors can substitute for serum in maintaining proliferation of hNSC and mimic the developmental sequence of growth factor dependence for hNSC (Kitchens et al., 1994; Teng et al., 2001).

Once a population of hNSCs was expanded, suspensions were plated on uncoated tissue culture dishes in growth media (Dulbecco's Modified Eagles Medium (DMEM) + F12 (1:1) supplemented with N2 medium (Gibco, Grand Island, NY), 10ng/ml leukemia inhibitory factor (LIF), 20ng/ml bFGF, 8 μg/ml heparin, and 20 ng/ml EGF. Growth media was changed every 5 days. Cultures were grown in monolayer. Any cell aggregates were dissociated in trypsin-EDTA (0.05%) when >10 cell diameters in size and were replated at 5×10^5 cells/ml. Four days prior to and continuing to the time of transplantation, both floating and adherent hNSCs were pre-labeled ex vivo with 20 μM bromodeoxyuridine (BrdU). On the day of transplantation, hNSCs were washed twice with PBS, dissociated with trypsin-EDTA followed by trypsin inhibitor, and rewashed with PBS before a final concentration adjustment and syringe loading. Cell viability was determined by trypan blue exclusion before implantation and again on the remaining cells after transplantation. Viability at both time points was found to be between 94–98%.

MPTP Lesioning and Transplantation

Seven adult, male St. Kitts green monkeys (*Chlorocebus sabaeus*) were examined in this study of NSC migration. Animals, housed at the St. Kitts Biomedical Research Foundation (St. Kitts, West Indies) were used in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the study was approved by the Institutional Animal Care and Use Committee.

Dopamine depletion and damage to SN dopamine neurons was induced using intramuscular injections of 1-methyl-4-phenyl-1,2,3,6-tetra-hydropyridine hydrochloride (MPTP). Monkeys were given 5 daily injections of 0.45 mg/kg MPTP over a 5 day treatment period for a total cumulative dose of 2.25 mg/kg. At 4 or 6 months after MPTP treatment, monkeys were implanted with hNSC bilaterally into the caudate, and unilaterally into the right SN for a total of three implant sites. Each implant site received approximately 1 million hNSC in a total volume of 10–20 μl and injected at a rate of 1 μl per minute. All animals were immunosuppressed with cyclosporine (0.6 mg/kg). Brains of the hNSC-implanted animals were collected at 4 months (n=3) and 7 months (n=4) post-implant. All animals were overdosed with pentobarbital to the loss of corneal reflexes and perfused with ice-cold physiological saline and then 4% paraformaldehyde. Brains were extracted and fixed for an additional 12 hours in paraformaldehyde. Brains then were stored in 30% sucrose buffer at 4°C until sectioning.

Immunohistochemistry, Cell Counts, and Statistics

Parasagittal frozen brain sections, 50 μm thick, were obtained using a sliding blade microtome. Sections were taken every 200 μm for free-floating, double-label immunohistochemistry. Anti-BrdU (BD Biosciences 1:50) was used to identify the BrdU incorporated in the hNSC during the incubation period prior to transplantation. Diaminobenzidine-nickel (DAB-nickel) was

used to color the hNSC black. Anti-tyrosine hydroxylase (TH; Chemicon, 1:1000) was used on the same sections to identify neurons expressing TH. DAB without nickel was used to color the TH+ neurons brown. In some adjacent sections fluorescent chromagens, rather than with DAB, were used to help identify double-labeled cells. In these sections BrdU was labeled with Texas red (Vector Laboratories) and TH was labeled with fluorescein (FITC: Vector Laboratories). Lastly, some sections were also double labeled for BrdU (DAB-nickel) and for glial fibrillary associate protein (GFAP; DAB only) to identify astrocytic differentiation. Four brain locations were studied to determine the strength and direction of hNSC migration (Figure 1). Three of the locations are specifically associated with the nigrostriatal dopamine system and included the caudate nucleus, the anterior portion of the nigrostriatal pathway (striatal end), and the posterior portion of the pathway including the SN (nigral end). The fourth location was in the thalamus dorsal to the SN. The thalamus was chosen as a control area because it is an area independent of the nigrostriatal system, is within the same sectioning plane, and has a similar parachymal composition to the striatum. If the hNSCs simply migrated in a non-directed fashion from the implant site or preferentially migrated up the implantation track, then the thalamus would have an equal opportunity to "host" the NSCs as the caudate nucleus and SN because all areas were penetrated by the implantation needles.

Migration of the hNSCs was identified by the number of BrdU positive cells and the dorsal-ventral / rostral-caudal location of each cell for each of the 4 locations. A modified unbiased optical fractionator method was used (King et al., 2002). Starting at an anatomically similar level for all animals, 5 brain sections per side were taken at approximately 600 μm intervals each. A photomontage was created for each of the 4 locations for each brain section and each side, creating 40 montages per animal. The pictures used to make each montage were taken at 20×, a magnification sufficient to identify both BrdU and TH positive cells. A grid, made of 400×400 μm counting frames, was superimposed onto the montage. The number of BrdU positive cells was determined in every other counting frame. Estimated total cell numbers (N) were determined as the number of cells counted (ΣQ) multiplied by the inverse of the area sampling fraction (1/asf) multiplied by the reference area (r.a.). The reference area was determined as the mean area divided by the section's actual area. Mean areas for each location were 22.25 mm^2 in the caudate, 34.08 mm^2 in the thalamus, and 52.02 mm^2 for both ends of the nigrostriatal pathway (Figure 1). Thus, the formula used for estimating total cell numbers was: N= (ΣQ) * 1/asf * r.a. Because of the jagged edge of implant tracks in general, which can "trap" antibodies and chromogens, creating false positive cells, positive cells in the implant tracks penetrating the caudate, SN, and thalamus were not used in our calculations for total cell numbers to prevent an artificial inflation of cell number. Because data regarding each counting frame was maintained, a contour map of the distribution and mean cell density per 400×400 μm frame was created. Estimates of total cell numbers were analyzed statistically using ANOVA and Fisher LSD post-hoc analysis with a significance level of $p < 0.05$. All statistics and graphing were done using Statistica 6.0 software (Statsoft, Inc.). Data from these animals, regarding changes in *endogenous* TH positive neurons of the striatum and the SN as a result of MPTP lesioning and hNSC presence have been reported elsewhere (Bjugstad et al., 2005; Redmond et al., 2007). This study focused principally on migration.

Results

Systemic MPTP administration to Old World monkeys creates the most authentic model available for PD in humans. We implanted undifferentiated human NSCs (hNSCs) into MPTP-lesioned, dopamine-depleted non-human primates in order to analyze their spontaneous migratory patterns in this pathological environment. Hypothesizing that these patterns were directed and non-random, we assayed these patterns at 4 and 7 months following grafting. Of the three million hNSCs that were implanted in each MPTP-lesioned monkey, between 180,000 and 340,000 donor-derived cells (i.e., BrdU-prelabeled cells) were detectable in the four

representative regions counted. Table 1 shows the percent of cells originally implanted that were found in each of the four areas (see Methods) and the total number of cells found. These areas were significantly different [$F_{(3, 30)}= 27.00$, $p < 0.0005$]. Interestingly, the caudate nucleus, which was implanted bilaterally with 2/3 of the hNSCs, had significantly fewer hNSCs than the areas surrounding the nigrostriatal pathway and SN [$p<0.05$]. In fact, there was no significant difference in cell numbers between the caudate nucleus and the unimplanted thalamus, suggesting that hNSCs had migrated from the implanted caudate [$p> 0.05$]. While not quantified, the putamen also had few BrdU positive cells. At least 80% of the counted BrdU-positive cells were found bilaterally along the nigrostriatal pathway and in the SN. More BrdU-positive cells were detectable along the nigrostriatal pathway after 7 months *in vivo* than at 4 months [$F_{(3,30)}= 2.90$, $p< 0.05$] (Figure 2). No significant differences in the number of BrdU-positive cells were found between the left/unimplanted SN and the right/implanted SN [$F_{(3,30)}= 0.40$, $p> 0.05$], suggesting that the hNSC migrated either from the ipsilateral implanted caudate nucleus to the SN, or from the contralateral SN. Figure 3 shows in overview the BrdU-positive cells (black nuclei) localized along the nigrostriatal pathway just posterior to the striatum, midway along the nigrostriatal pathway, and within the SN. Very few BrdU-positive cells were seen in the caudate nucleus (where the cells were initially implanted), the thalamus, or other surrounding brain areas. There were no significant differences between the 4 month and 7 month animals in the number of BrdU-positive cells found in the caudate or the thalamus.

To visualize better and more quantitatively the distribution of donor-derived cells based on the mean density of the BrdU+ cells, contour mapping plots were generated according to the coordinates of the stereological counting frames (see Methods). Analyses of these maps indicated that there were significant changes in the density of hNSC-derived cells as one moved from dorsal to ventral and from rostral to caudal for both the striatal half of the pathway and the nigral half of the pathway [$F_{(20, 252)}= 3.83$, $p< 0.0001$ and $F_{(20,252)}=1.85$, $p < 0.05$ respectively] (Figure 4). The greatest density of donor-derived cells found in the striatal halves of the pathway corresponded to the area located ventral and caudal to the anterior commissure ("ac") for both 4-month-post-grafting and 7-month-post-grafting groups. There were significantly fewer donor-derived cells in the regions in anterior, dorsal, and ventral to this area ($p < 0.05$). In the nigral half of the pathway, the highest density of BrdU-positive cells corresponded to the SN in both groups. Areas dorsal and posterior to the SN had significantly fewer cells ($p < 0.05$). The density distribution pattern suggested that most of the hNSCs migrated to or along the location of the nigrostriatal pathway and SN.

Double label immunohistochemistry, using both DAB and fluorescence labels, was used to determine if the NSC differentiated into dopamine neurons or astrocytes. In this set of animals, no TH positive neuron in the SN was found to have a BrdU positive nucleus (Figure 5) In addition, no cells that double-labeled for BrdU and TH were seen in the striatum (data not shown). Co-staining for GFAP revealed brain regional differences. While few BrdU labeled cells were found in the striatum, those that were found there, occasionally were colabeled with GFAP, suggesting a differentiation into astrocytes (Figure 6). A similar phenomena was seen along the nigrostriatal pathway, in particular the area ventral to the anterior commissure. In the SN, there were no BrdU-positive cells which also labeled for GFAP in either group. The hNSC appeared to remain as undifferentiated neural progenitors.

Discussion

The present study contributes to our understanding of stem cell behavior by affirming for the first time that NSCs of human origin can move over appreciable distances to regions of pathology in a non-human primate brain that models the complexity of human neurodegenerative disorders, in this case PD. While the directed migration of NSCs is certainly

not a new concept, no one study, to our knowledge, has pieced together all the important elements described above that might provide the confidence to believe that the cell-based strategies we and others have proffered might be feasible for patients.

Nearly all (~80%) of the hNSC identified 4–7 months following transplantation into parkinsonian monkeys, distributed in a non-random fashion along the nigrostriatal pathway and within the SN. Although the hNSCs had been implanted unilaterally into the SN, by the time of sacrifice 4 or 7 months later, hNSCs were found bilaterally in the SN. The caudate nucleus, which had been implanted bilaterally, ultimately harbored no more hNSC than the thalamus, a structure not directly affected in parkinsonian degeneration and not implanted with hNSC. These data suggest that hNSC migrate preferentially to the regions of cellular loss or impairment after MPTP administration in the monkey. Again, as noted above, we believe this study to be the first quantification of damage-directed NSC migration in non-human primates, providing greater clinical relevance to previous such reports in rodents (Aboody et al, 2000; An et al., 2004; Brundin et al., 2003; Chu et al., 2003; Ishibashi et al., 2004; Jeong et al., 2003; Kelly et al., 2004; McBride et al., 2004; Pluchino et al., 2003; Redmond et al., 2007; Tang et al., 2003; Wennersten et al., 2004).

There may be several explanations for the fact that only ~6–11% of the total number of hNSCs transplanted could be identified after 4–7 months. That only a tenth of the cells were detectable may be attributable in part to *methodological* limitations. For example, pre-incubation of hNSCs, which cycle much slower than their murine counterparts, with BrdU *ex vivo* pre-labels only those hNSC entering S-phase during the 4 days of exposure. It typically takes at least a week for all hNSC in a dish to cycle completely. Thus only a portion of the transplanted hNSC will have intercalated BrdU into their genome rendering the others, while present, "invisible" to BrdU immunohistochemical detection. Reassuringly, the hNSC integrate into the host cytoarchitecture with no graft margins, overgrowth, or distortions, making them indistinguishable from host cells. Other explanations for finding only a tenth of implanted hNSC might be *biological:* (1) The engraftable "niche" may only have "required", or been able to accommodate, a portion of the hNSCs supplied, leaving the others non-integrated (in other words, our cell "dosage" was higher than required); (2) hNSCs may have integrated, but then died, either from lack of connectivity, from normal pruning processes, or by immunorejection (despite aggressive immunosuppression). Nevertheless, important conclusions emerged from the hNSC that could be tracked and quantified.

That few, if any, of the subpopulation of detectable donor-derived cells differentiated into TH + neurons, despite being alligned along the nigrostriatal pathway, is consistent with previous reports (Ishibashi et al., 2004; Jeong et al., 2003; Kelly et al., 2004; Li et al., 2003; McBride et al., 2004; Ourednik et al., 2002; Sun et al., 2003; Wennersten et al., 2004). Neuronal differentiation requires a long maturation process, even for endogenous neural progenitors in normal neurogenic zones of a rodent (Song et al, 2002). Because of our focus on migration, this study using human cells (a species that is already slow to differentiate) in a monkey brain (which supports only slow neuronal differentiation) ended after only 4 or 7 months. Our previous study (Redmond et al., 2007), however, did allow transplanted undifferentiated hNSCs to mature longer *in vivo* in some monkeys. In that study a subpopulation of donor-derived cells did ultimately co-express TH and the dopamine transporter (DAT), although they constituted a minority (<1%) of the donor-derived cells that subpopulation made a sizeable contribution to the total TH population of the parkinsonian monkeys' SN. That NSC not pre-differentiated towards a neuronal lineage *ex vivo* (prior to engraftment) yield principally *non*-neuronal "chaperone" cells *in vivo* is emerging as a prevailing finding in many animal models of neurodegeneration, including PD. The requirements for non-neuronal "chaperone" cells may also be region specific. We found that hNSC did differentiate into astrocytes in the caudate and in the striatal end of the nigrostriatal pathway, but hNSC found in the SN as undifferentiated

quiescent neural progenitors, suggesting that the cells are meeting different needs in different areas. The recognition is that non-neuronal NSCs attempt to restore homeostasis to a disordered system by providing neurotrophic and neuroprotective molecular support (Lu et al., 2003; Ourednik et al., 2002; Yan et al, 2005; Llado et al, 2005), by detoxifying the milieu (Llado et al, 2005; Taylor et al, 2006), by inhibiting inflammation and gliotic scarring (Teng et al, 2002; Park et al, 2002; Pluchino et al., 2003; Lee et al, 2007), by laying down supportive extracellular matrices, and by restoring intraneuronal molecular equilibria (Ourednik et al, 2002; Redmond et al, 2007; Li et al, 2006). From that perspective, for a stem cell to migrate along the neural pathway affected by a given disease in order to enhance its juxtaposition with the perikaryon and axonal projections of endangered neurons, as seen presently, makes teleological sense. It is intriguing that the hNSCs seem to optimize their proximity by recapitulating at least one of the proposed migratory paths employed during embryonic development of the nigrostriatal system.

Differentiated hNSC-derived neurons do not appear to engage in providing such molecular support or participate in this extensive migration (Lu et al, 2003; Yan et al, 2005; Llado et al, 2005; Burnstein et al., 2004; Wang et al., 2004). Indeed, the literature supports the concept that the most migratory (and potentially therapeutically efficacious) cells are those that are the *least* differentiated at the time of transplantation. In earlier reports on fetal neural tissue transplantation, it was appreciated that grafts consisting of more differentiated cells did not migrate over great distances (Harrower et al., 2004; Sladek et al., 1993), although neurite extension may occur for several millimeters (Giacobini et al., 1993; Sladek et al., 1993; Wang et al., 1994). More recently, NSC transplantation studies similarly have found that undifferentiated NSCs have a greater tendency to migrate and survive than do NSCs pre-differentiated *ex vivo* (Burnstein et al., 2004; Wang et al., 2004; Le Belle et al., 2004; Yang et al., 2004). Sun et al. (2004) present data suggesting that undifferentiated, but not differentiated, NSCs express the stem cell factor (SCF) receptor, c-kit, which allows them to migrate to damaged tissues expressing SCF, a migration that can be inhibited by c-kit blockade. In 6-OHDA-lesioned rats, NSC pre-differentiated to express neuronal markers *ex vivo* when transplanted into the striatum failed to promote behavioral recovery despite the fact that they continued to express neuronal markers (Burnstein et al., 2004).

Accordingly, as noted here, as well as in our prior reports documenting functional recovery in both rodent and primate PD models (Ourednik et al., 2002; Bjugstad et al, 2005; Redmond et al, 2007), most NSCs remained either undifferentiated or pursued a non-neuronal lineage (Figure 5). In our earlier report (Bjugstad et al., 2005), we found that the MPTP-induced changes in the size and number of endogenous TH-positive neurons of the striatum and SN were reversed in the 4 month and 7 month animals (Betarbet et al., 1997; Bezard et al., 1998; Dubach et al., 1987; Elsworth et al., 1996; Porritt et al., 2000; Redmond et al., 2007). This effect was plausibly linked to the production of growth factors (such as GDNF) by non-neuronal hNSC-derived cells (Redmond et al, 2007; Ourednik et al, 2002). Taken together, it would appear that implanting hNSCs in an undifferentiated, but migratory state optimizes their ability to exert homeostatic actions across the disequilibrated nigrostriatal system. Conversely, non-migratory pre-differentiated hNSCs appear to play a more discrete (even limited) clinical role, since they would need to be placed directly into specific loci of damage.

We have no data at this point to determine what the attractants are that directed hNSC migration in the MPTP model. An on-going inflammatory response in the SN and nigrostriatal pathway might explain the migration to these areas (Imitola et al, 2004), especially in light of recent evidence that the MPTP lesion in primates has a sustained and progressive inflammatory signature (as does PD in humans). Alternatively, some of the numerous genetic and epigenetic mechanisms that direct cellular migration during development might be recapitulated by this

degenerative process as they are in others (Snyder et al, 1997; Park et al, 2006). Future studies will attempt to discern the signals that dictate these migratory patterns in Parkinsonism.

In summary, we found that undifferentiated hNSCs, when implanted into adult MPTP-lesioned parkinsonian primates, spontaneously migrated in a non-random, directed fashion from their point of implanation to align themselves along and within the impaired nigrostriatal pathway. Given that hNSCs exert some of their most potent therapeutic actions as "chaperone" cells providing molecular support, the ability of hNSCs to home to pathological zones (even in the adult primate brain) and to recapitulate developmental migratory patterns, allows this cellular strategy to potentially offer an important adjunct to other PD therapies that might operate within more discrete loci. This study represents one of the first demonstrations that human stem cells can migrate extensive distances even in the adult primate brain, providing a level of confidence that the strategies we have previously discussed might be applicable to patients (Bjugstad et al, 2005; Redmond et al, 2007).

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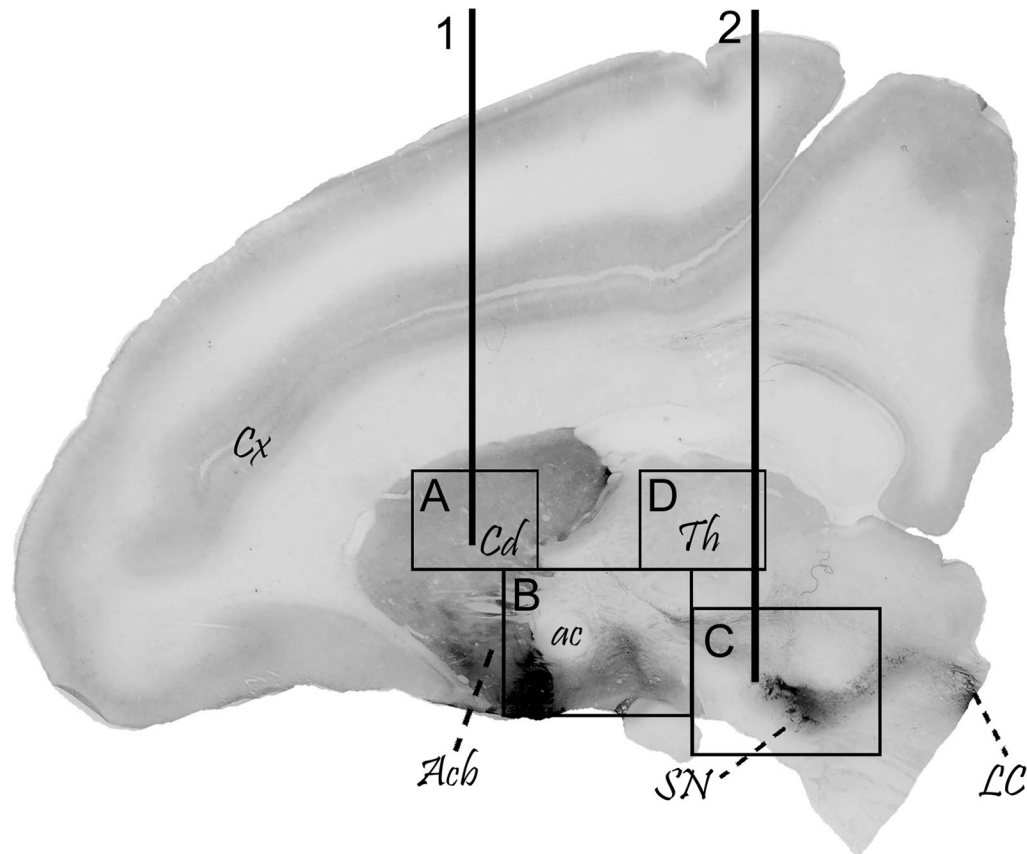
FIGURE 1.
Parasagittal section of a monkey brain, with boxes depicting the four areas in which counts of BrdU+ cells were made. The caudate nucleus (A) was implanted bilaterally (line 1) with BrdU pre-labeled hNSCs, while the SN (C) was implanted unilaterally only (line 2). Most BrdU-positive cells appeared in the areas between the caudate and the SN, along the nigrostriatal pathway (boxes B and C). Cell counts were done for the anterior portion of the pathway (B: Striatal Half) and for the posterior portion of the pathway, which included the SN (C: Nigral Half). The thalamus (D) also was included as a control area which is not a component of the nigrostriatal dopamine system, although the SN implant track (line 2) penetrated the right thalamus. This parasagittal section was immunostained for tyrosine hydroxylase (TH, black immunoreactive regions) to better depict the SN, the nigrostriatal pathway, and innervation of the CD. Cx- cerebral cortex, Cd- caudate, ac- anterior commissure, Th- thalamus, SN-substantia nigra, LC- locus coeruleus.
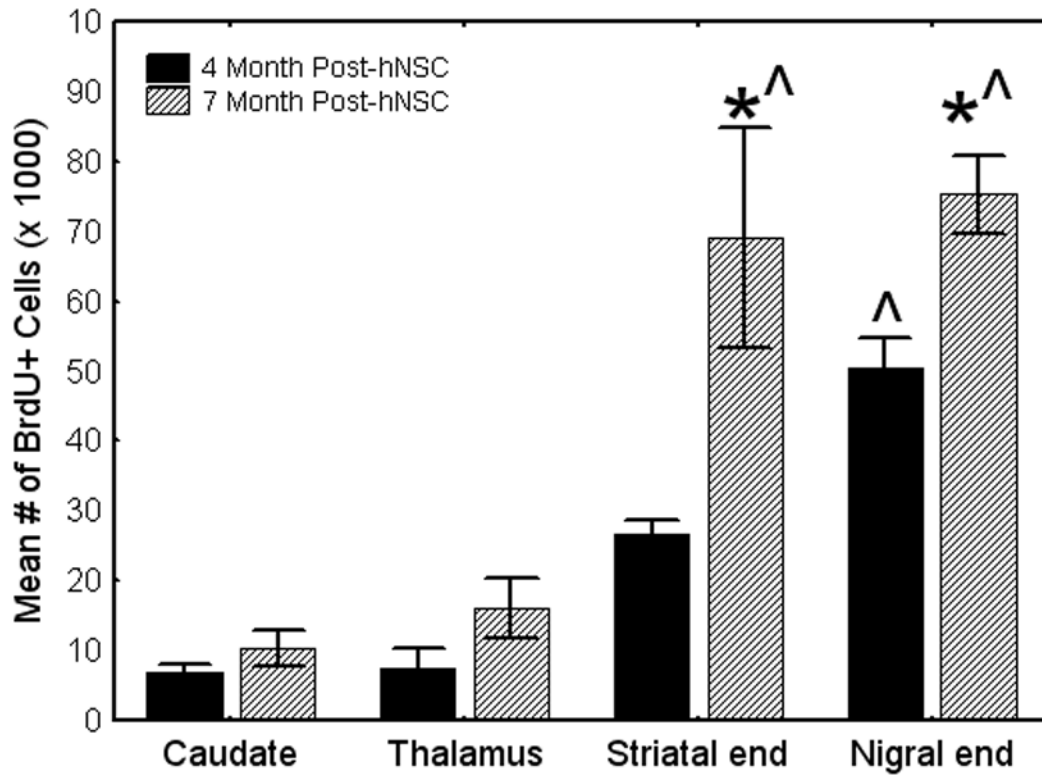
FIGURE 2.
The total number of BrdU-positive cells found in each area for MPTP-lesioned monkeys 4 months and 7 months post-transplantation. There were significantly more BrdU positive cells found along the nigrostriatal pathway (striatal end and nigral end) than in the caudate nucleus, an area specifically implanted with hNSC [F(3, 30)= 27.00, p< 0.0005]. As many cells were found in the thalamus, an unimplanted site, as were found in the caudate nucleus (p> 0.05). The 7 month animals had significantly more BrdU+ cells along the nigrostriatal pathway than the 4 month animals [F(3,30)= 2.90, p< 0.05]. There was no difference between the groups in the number of cells found in the caudate and thalamus. * Significantly greater than the same region in the 4 month animals. ^ Significantly different than the other brain areas. Significance level p< 0.05.
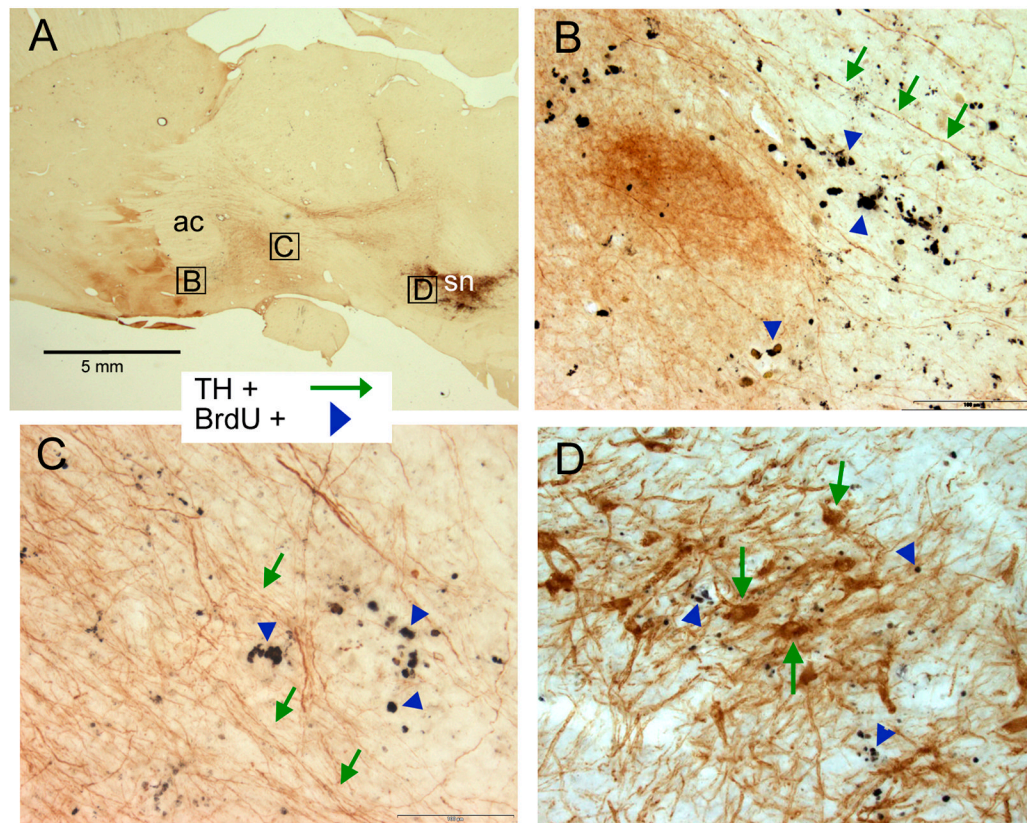
FIGURE 3.
Overview the nigrostriatal pathway and the BrdU-positive hNSC-derived cells found there. hNSCs were labeled with BrdU *ex vivo* prior to transplantation. Immunohistochemistry against BrdU and TH was used to identify the location of the hNSCs (black nuclei) and the dopamine neurons and their fibers (brown), respectively. [A] A low power view of the nigrostriatal pathway illustrating where the photomicrographs of [B-D] were taken. The anterior commissure (ac) and substantia nigra (sn) are labeled. [B] Anterior portion of the nigrostriatal pathway. hNSCs (black nuclei, blue arrowheads) were found ventral to the anterior commissure along with TH positive fibers originating from the midbrain (brown fibers, green arrows). [C] Posterior portion of the nigrostriatal pathway. BrdU-positive cells were found (black nuclei, blue arrowheads) along with many TH-positive fibers (brown, green arrows). [D] The endogenous TH+ neurons of the SN (brown, green arrows) were surrounded by BrdU-positive cells (black nuclei, blue arrowheads). A *density contour map* of these cells is shown in Fig. 4 and a higher-power view using dual immunofluorescence of the relative positions of donor hNSC-derived cells and host TH+ cells is presented in Figure 5.
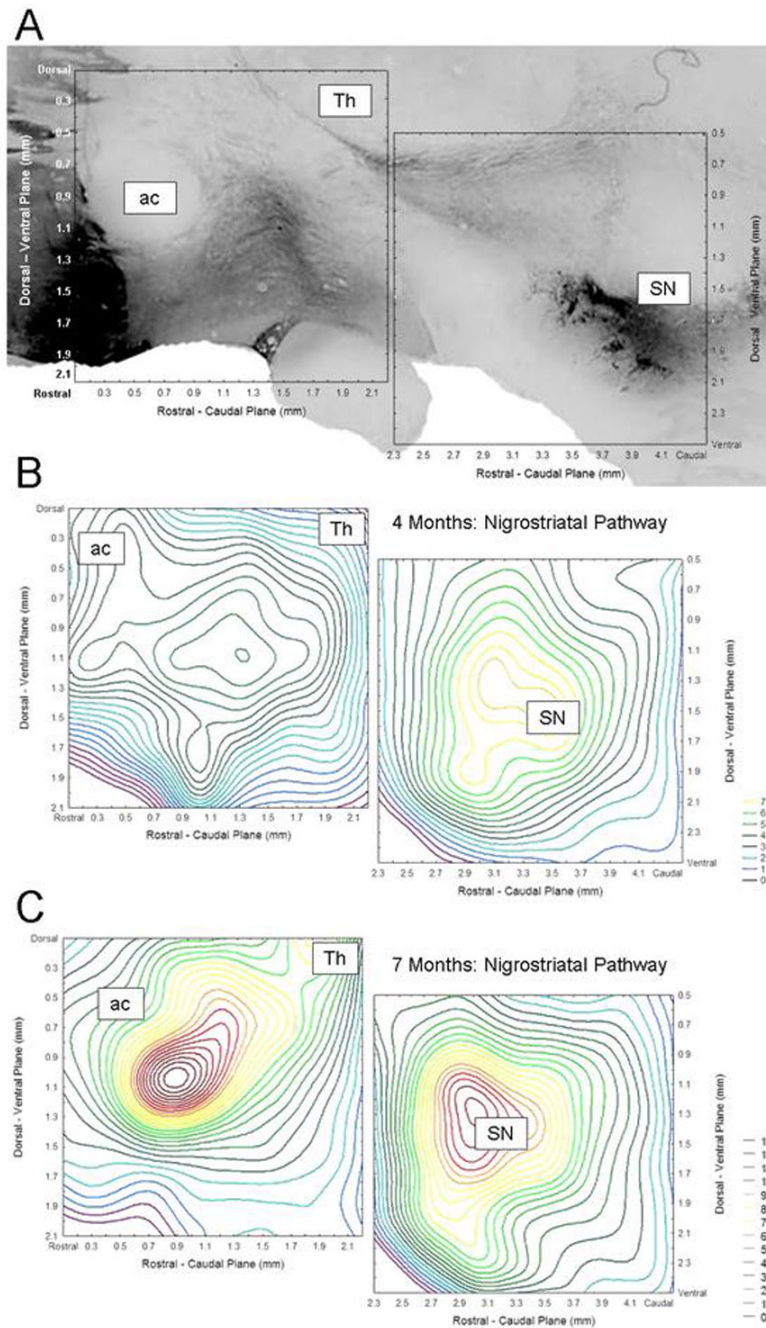
FIGURE 4.
hNSC density contour mapping. The mean number of BrdU-positive cells at each dorsal-ventral / rostral-caudal location (counting frames of 400×400 μm) was plotted to create a hNSC density contour map to show the changes in density of hNSC across the two planes. [A] An overlay of the X and Y axis onto a photomicrograph of the areas being described. [B] Contour mapping of the striatal and nigral half of the nigrostriatal pathway for the 4 month-post transplant data. The greatest density of hNSCs was found in the SN with >7 BrdU+ cells per 400 μm^2 (yellow contour lines). [C] By comparison, the 7 month post-transplant data had a high density of BrdU+ cells at the posterior/ventral side of the ac and in the SN, with almost

twice the density (red contour lines). ac= anterior commissure, Th= thalamus, SN= substantia nigra.
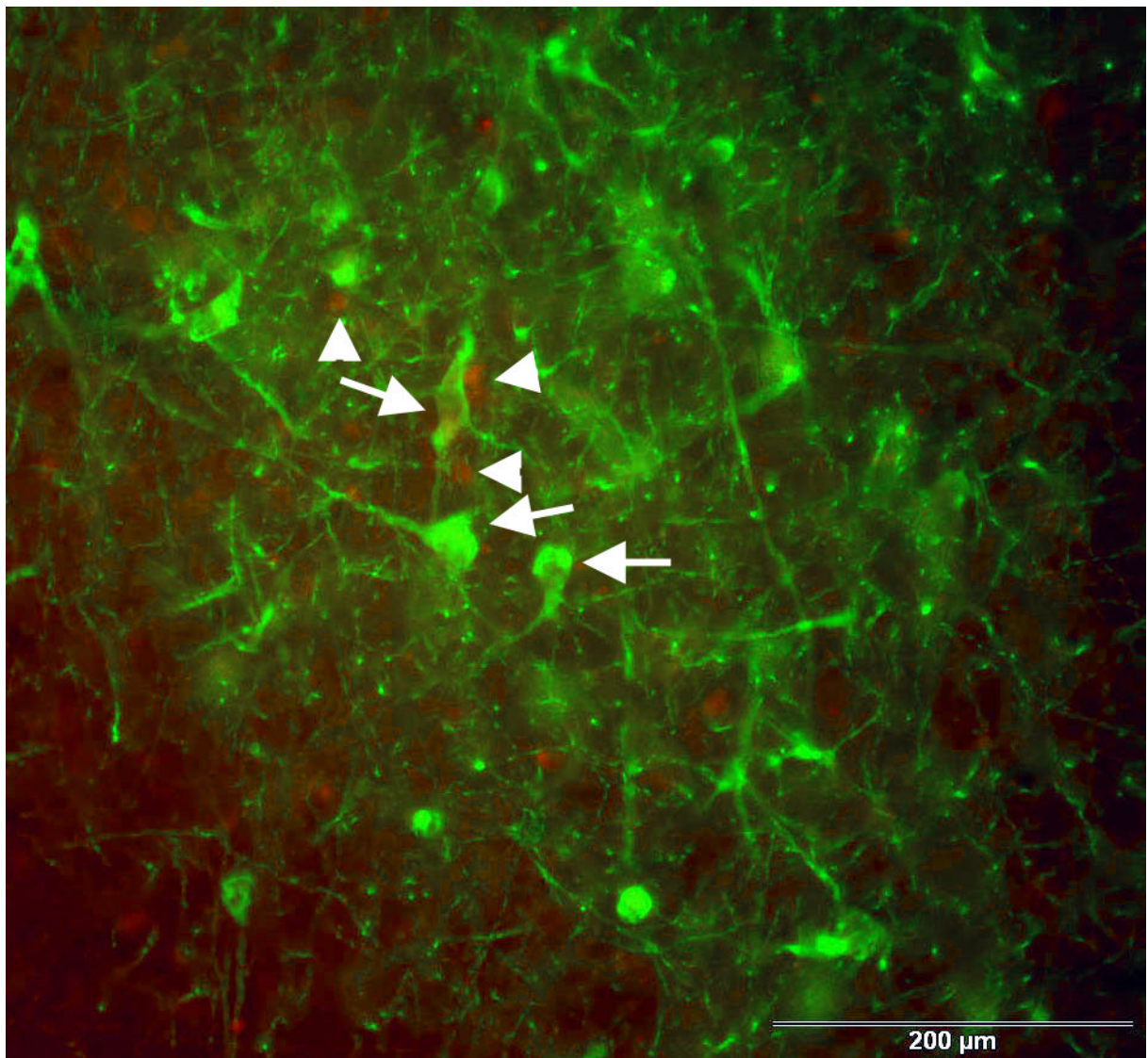
FIGURE 5.
Dual immunohistochemistry for BrdU+ and TH+ cells demonstrating the intimate association between donor hNSC-derived cells (red nuclei, arrowheads) and host SN dopaminergic neurons (green somata and processes, arrows), respectively. Note that no TH+ neurons in the SN co-labeled with BrdU, suggesting that dopaminergic lineage was not the preferred fate of these undifferentiated hNSCs in the SN.
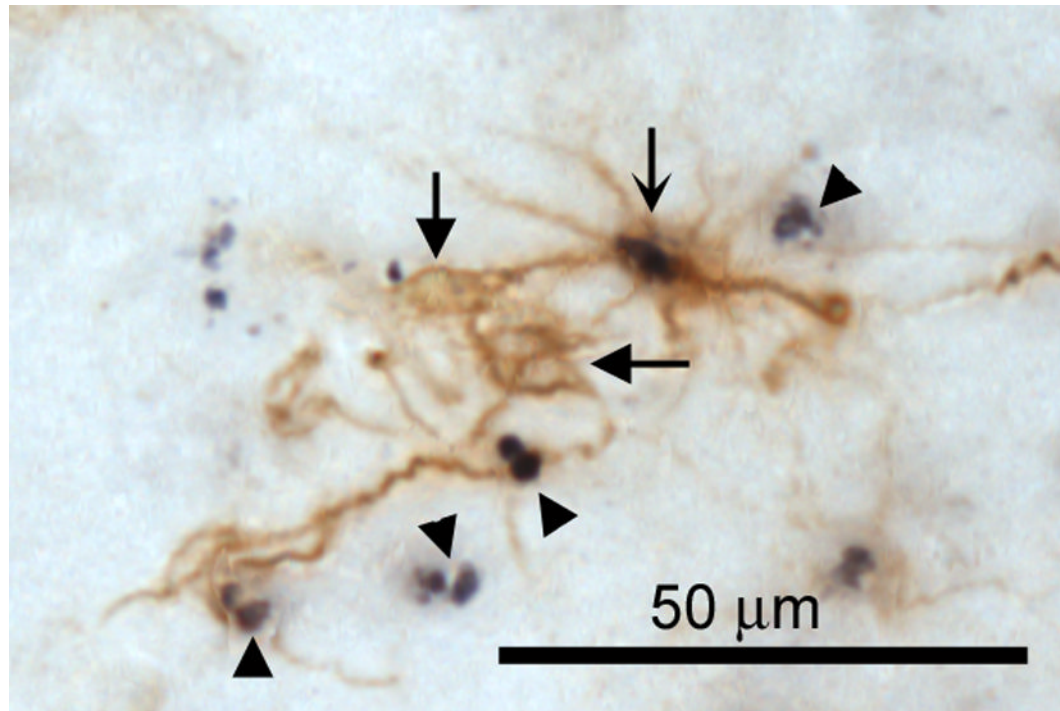
FIGURE 6.
Some striatal astrocytes double-label for BrdU suggesting that they are of donor origin. GFAP
was used to identify astrocytes (brown) while BrdU was used to identify the implanted hNSC
(black nuclei). Most astrocytes did not double label for BrdU (brown cells, white nucleus,
closed-headed black arrows). The nuclei of these cells are void of BrdU labeling. An occasional
astrocyte appeared with a BrdU labeled nucleus (brown cell with black nucleus, open-headed
arrow), however most BrdU labeled cells did not co-localize with GFAP (black nuclei, black
arrow heads).

Table 1

The percent of hNSCs found in each neuroanatomical area studied.

		% in Caudate $22.25\ mm^2$	% in Thalamus $34.08\ mm^2$	% in Striatal Half of Pathway $52.02\ mm^2$	% in Nigral Half of Pathway $52.02\ mm^2$
	Mean Area				
4 Months Post-Implant	Right (CD/SN)	0.27 ± 0.07	0.40 ± 0.14	0.94 ± 0.11	1.88 ± 0.16
	Left (CD only)	0.19 ± 0.03	0.11 ± 0.02	0.83 ± 0.12	1.49 ± 0.21
	Total			**6.10 ± 0.58**	
7 Months Post-Implant	Right (CD/SN)	0.40 ± 0.15	0.70 ± 0.27	2.85 ± 0.94	2.69 ± 0.26
	Left (CD only)	0.29 ± 0.09	0.38 ± 0.07	1.75 ± 0.46	2.34 ± 0.27
	Total			**11.38 ± 1.47** *	

The percentages represent the number of cells located as a percent of 3 million cells (1 million per implant site). Note that some hNSC were located in the left nigral half of the pathway for both 4 month and 7 month animals. Since no hNSCs were implanted into the left SN, this suggests that the hNSC migrated from the implanted/right SN or migrated caudally from the ipsilateral caudate nucleus. There was no significant difference in the number of BrdU-positive cells found between the right (double implanted) and left (single implanted) sides [F(1,10)= 3.40, p >0.05]. A significantly greater total number of BrdU positive cells were detected in the 7 month animals than in the 4 month animals [F(1, 10)= 12.60, p< 0.005]. Statistical details regarding specific areas are shown in Figure 2.

*
$p < 0.05$.