IN the current experiments, we address the emerging hypothesis that transplanted neural precursor cells can respond to local microenvironmental signals in the post-developmental brain and exhibit patterns of differentiation that depend critically on specific location within the brain. HiB5 precursor cells were transplanted into adult mouse cortex, corpus callosum, and multiple positions in striatum, and assessed for differentiation by morphology and immunocytochemistry. Our results indicate that the likelihood of both neuronal and glial differentiation of transplanted precursors depends on proximity to the medial striatum or subventricular zone of the adult host, supporting the concept that microenvironmental signals can critically affect the differentiation fate of neural precursors, and suggesting the potential to manipulate such signals in the adult brain. NeuroReport 10:3971–3977 © 1999 Lippincott Williams & Wilkins.

**Key words:** Conditionally immortalized cell line; HiB5; Glial differentiation; Microenvironment; Neuronal differentiation; Striatum; Transplantation

### Introduction

The possibility of repopulation therapies for neurodegeneration or injury in the mammalian CNS would require appropriate transplanted or endogenous precursor cells to functionally replace lost neurons. This in turn would require the diseased or injured brain to support the survival and differentiation of repopulating cells. Examining the fate of precursors transplanted into different regions of the brain in intact or perturbed hosts, at different stages of development, gives insight into the local microenvironmental signals that are required for the survival, proliferation, migration, differentiation, connectivity, and synapse formation of these precursors in new environments. Transplantation into adult animals has shown that certain regions of the intact adult brain, including striatum and hippocampus, are capable of supporting the neuronal differentiation of donor precursor cells [1–4] paralleling and consistent with the discoveries of the existence of endogenous precursor cells in these regions in adults [5–8].

The use of primary immature neurons or precursors in transplantation is limited somewhat because of heterogeneity and restricted availability. Conditionally immortalized precursor cell lines, such as the multipotential HiB5 cell line, developed by Renfranz et al. [9], largely avoid these limitations (although immortalization itself may introduce chromosomal instability, and therefore genotypic variability, in such cells). HiB5 cells, derived from E16 rat hippocampal precursors, were immortalized with a temperature-sensitive allele of the SV40 large T antigen. Renfranz et al. transplanted HiB5 cells into neonatal hippocampus or cerebellum, both still undergoing neurogenesis, and demonstrated that HiB5 cells integrate and assume morphologies characteristic of endogenous neurons and glia, establishing the multipotentiality of these cells in the developing cerebellum and in their homotypic hippocampal environment at young ages [9].

In the present study, we tested this hypothesis by explicitly examining the pattern of distribution of transplanted HiB5 cells in the striatum to determine if those that have differentiated into neurons are...
concentrated in the medial periventricular region. Our results indicate that the probability of neuronal or glial differentiation is greatest when the position of the donor cell in the striatum is most medial. These results, that the subventricular zone and/or medial striatum permit increased levels of neuronal and glial differentiation, support the concept that microenvironmental signals can critically affect the differentiation fate of neural precursors and suggest the potential to manipulate such signals in the adult brain.

Materials and Methods

Cell culture: HiB5 cells (gift of R.D.G. McKay) were incubated in 5% CO₂/95% air at 33°C on culture dishes (Falcon) precoated for 60 min with 15 μg/ml polyornithine (Sigma). The cells were cultured in serum-containing medium (Dulbecco’s modified Eagle’s medium (Gibco), 10% fetal calf serum (Gibco), 2.5% HEPES, 1% sodium pyruvate, and 1% penicillin–streptomycin). Medium was changed twice weekly and cells were passaged when 75–80% confluent. HiB5 cells were derived from E16 rat hippocampal precursors and immortalized with a temperature-sensitive allele of the SV40 large T antigen. Because chromosomal instability has been reported to be a concern in cells transfected with the SV40 large T antigen, we used early passage cells and observed no tumorigenesis following transplantation, consistent with a temperature-sensitive allele of the SV40 large T antigen. We used early passage cells and observed no tumorigenesis following transplantation, consistent with a temperature-sensitive allele of the SV40 large T antigen. We used early passage cells and observed no tumorigenesis following transplantation, consistent with a temperature-sensitive allele of the SV40 large T antigen. We used early passage cells and observed no tumorigenesis following transplantation, consistent with a temperature-sensitive allele of the SV40 large T antigen.

Preparation of non-viable control cells: Control cells were rendered non-viable by 6–8 successive freeze/thaw cycles in hypotonic medium (viability by trypan blue exclusion < 0.1%) to confirm that the results were specific to viable cell transplants. Transplants of lysed HiB5 cells prelabeled with [³H]thymidine and fluorescent markers did not show non-specific transfer of labels to endogenous cells.

Transplantation: Twenty-eight 6-week-old C57B/6J mice were deeply anesthetized with Avertin and transplanted with live or non-viable control HiB5 cell suspensions unilaterally or bilaterally at varying distances from the lateral ventricle using modified stereotaxic coordinates (Fig. 1A). Each injection into striatum, corpus callosum, and neocortex contained ~1 μl of 10⁴ cells/μl. All procedures were undertaken in accordance with federal and institutional guidelines after protocol review and approval.

Tissue processing: One to five weeks after transplantation, mice were deeply anesthetized with Avertin and transcardially perfused with saline followed by 4% paraformaldehyde. Brains were removed, post-fixed for at least 12 h in 4% paraformaldehyde, and coronally sectioned using a vibrating microtome (Vibratome) at 100 μm for three-dimensional PKH or DiI analysis and reconstruction, and at 40 μm or 50 μm for immunocytochemistry.

Immunocytochemistry: Sections from each animal were processed with glial fibrillary acidic protein (GFAP; Incasta) to identify astroglia, and one or more of the following antibodies to identify neurons: microtubule-associated protein 2 (MAP2; Sigma), the mature neuronal transcription factor NeuN (gift of R. Mullen, University of Utah), and neuron-specific enolase (NSE; Zymed). Sections from experiments involving BrdU-labeled cells were processed with BrdU-specific antibody (Harlan). For
autoradiography studies (MAP2, NSE, GFAP) sections were pretreated with Immunopure Peroxidase Suppressor (Pierce) to quench endogenous peroxidase activity, blocked in medium containing 5% bovine serum albumin, 3% goat serum and 0.3% Tween (Sigma), incubated in primary antibodies (diluted 1:500, 1:100, 1:50 and 1:100, respectively), washed, and incubated in secondary antibodies (Alexa 546 for MAP2 and NeuN, 1:500; Alexa 350 for GFAP, 1:100; Alexa 488 for BrdU, 1:250).

**Autoradiography:** Following immunocytochemistry, sections were mounted on coated glass slides, dehydrated, and dipped in NTB-2 emulsion (Eastman Kodak) for autoradiography. Sections were developed after 4–6 weeks of exposure. Variable incubation times did not produce significant differences in background density.

**Quantification of autoradiography and immunocytochemistry:** For quantitation of cell differentiation, transplanted HiB5 cells were identified by [3H]-thymidine labeling. In order to eliminate the possibility of false positive identification of transplanted cells, we used high numerical aperture optics with shallow depth of focus; as [3H]thymidine emission penetrates only several microns through tissue [18], cellular identification is quite accurate. Furthermore, we only counted cells that were very heavily labeled with [3H]thymidine; cells were recorded as [3H]thymidine-labeled only when the number of silver grains over the nucleus of the cell exceeded the average background grain density by 3 s.d. Although background silver grain density was consistently low, and variable incubation times did not produce significant differences in background grain density, labeling of a cell was evaluated by comparison with background on the same tissue section to optimize reliable donor cell identification most conservatively.

The fraction of heavily labeled transplanted cells which underwent differentiation as assessed by immunocytochemistry was calculated by dividing the number of double-labeled cells (both antibody-labeled and heavily [3H]thymidine-labeled) by the total number of heavily labeled [3H]thymidine-positive cells for each section. This differentiation fraction was normalized, with the value corresponding to the mid-lateral position in the striatum (500 μm from the lateral ventricle) defined as 1.0. The overall percentage of differentiated HiB5 cells, 6 weeks after transplantation into the striatum, has been previously reported by Lundberg et al. [2] to be approximately 3% for neuronal differentiation and 8% for glial differentiation; our data are consistent with their findings.

**Statistical analysis:** For neuronal differentiation
analysis, data from nine independent transplantation experiments were pooled (total number of cells counted = 1892); analysis of glial differentiation combined data from eight independent transplantation experiments (total number of cells counted = 1536). For each \(^{3}H\)thymidine-labeled cell, lateral position of the cell (distance from the lateral ventricle) and immunolabeling were recorded. Data were analyzed by direct logistic regression analysis. This analysis produces an exponential function to describe the relationship between two variables (in this case, cell position and immunolabeling) and calculates a \(p\) value to describe the significance of the relationship between the actual data and the proposed function [19] (David Zurakowski, PhD, institutional statistician, personal communication; Dianne Finkelstein, PhD, Massachusetts General Hospital Biostatistics Center, personal communication).

**Results**

*Migration of transplanted cells as a function of region of transplantation:* Cells dispersed to the greatest degree within the corpus callosum, to an intermediate degree within the striatum, and least within the neocortex. Donor HiB5 cells, prelabeled with fluorescent microspheres and the lipophilic dye PKH or DiI, dispersed only \(~100\mu m\) within cerebral cortex (Fig. 1B,C). Within corpus callosum, cells dispersed up to \(1\) mm laterally (Fig. 1D,E). Within the striatum, cells dispersed up to \(400–500\mu m\) laterally (Fig. 1D,E).

*Differentiation of transplanted cells as a function of region of transplantation:* Donor cells identified by PKH, DiI, or fluorescent microsphere labeling differed markedly in morphology depending on their location. Cells remaining at the injection site were large (average cell body size \(=120\mu m^2\)) and round, retaining the morphology of undifferentiated cells prior to transplantation (data not shown). In contrast, transplanted cells in cerebral cortex were typically spindle-shaped and glial in morphology, with 1–3 processes extending from the cell body, and were distinctly smaller (average cell body size \(=25\mu m^2\)) than those seen in adjacent regions (data not shown). In the corpus callosum, donor cells were small and bipolar, suggestive of glial morphology (Fig. 2D). In contrast, in the striatum, a fraction of donor cells acquired the morphological appearance of neurons, not seen in other regions of transplantation, with elaborate processes and large cell body size (Fig. 2A–C).

The neuronal differentiation of HiB5 precursor cells in striatum was confirmed by immunocytochemical analysis. A fraction of donor cells was positive for neuronal markers: MAP2 (Fig. 3A–C), NeuN (Fig. 3D–G) and NSE (not shown), or for
the astroglial-specific marker GFAP (Fig. 3H). Immunocytochemical co-localization studies were performed to investigate whether the cells expressing neuronal markers and those expressing astroglial markers were separate populations, or whether differentiating HiB5 precursors could co-express neuronal and glial markers. Donor cells were immunostained with antibodies against BrdU (Fig. 3I,M), GFAP (Fig. 3K,N) and MAP2 (Fig. 3J) or NeuN (Fig. 3O). All MAP2- and NeuN-positive donor cells were GFAP-negative ($n = 87$ and $n = 44$, respectively; Fig. 3L) and all GFAP-positive donor cells were MAP2-negative ($n = 53$) and NeuN-negative ($n = 97$; Fig. 3P). These results, that differentiating HiB5 cells do not co-express neuronal and astroglial markers, demonstrate that those HiB5 cells differentiating into neurons and those differentiating into glia are separate populations.

**Specific location-dependence of differentiation**: The fraction of surviving, heavily labeled precursors which differentiated into neurons following transplantation varied significantly with location in the striatum (Fig. 4A). The neuronal normalized differentiation fraction (DF), which reflects the fraction of heavily $[^{3}H]$thymidine-labeled HiB5 cells which were MAP2-positive, varied from 1.5 medially (<100 $\mu$m from the lateral ventricle), to 0.8 laterally (>900 $\mu$m from the lateral ventricle). Glial differentiation was also dramatically greater in the medial...
We investigated the pattern of neuronal and glial differentiation of HiB5 multipotent precursors following transplantation into three different regions of the intact adult brain (neocortex, corpus callosum and striatum), and, specifically, into multiple medio-lateral positions within the striatum. Comparison of the fate of cells transplanted into cerebral cortex, corpus callosum and striatum reveals that, of these, only striatum supports the neuronal differentiation of donor cells. In addition, our data demonstrate that, within the striatum, the medial periventricular striatum supports both neuronal and glial differentiation to a greater degree than do the further lateral regions of the striatum. This finding suggests that the adult periventricular region constitutes an environment that, unlike most other regions of the adult brain, contains signals for the differentiation of neurons from precursor cells.

However, experiments using cells with such variable chromosomal complement and potentially variable genotype must be interpreted with caution. Chromosomal number in excess of diploid raises the possibility that there may be variable numbers of important genes involved in differentiation or phenotype analysis (e.g. GFAP, MAP2, NeuN), and that this variability could confound analysis of the differentiation state of these cells. The potential exists that local environmental factors, rather than solely modifying the differentiation of a homogeneous clonal population, may be selectively affecting survival and/or differentiation of somewhat heterogeneous precursors. Thus, prior studies demonstrating specific differentiation fates using HiB5 cells and other similarly immortalized cell lines may be confounded in their interpretation by these complexities.

A number of studies in recent years have demonstrated region-specific differentiation of neural precursor cells following transplantation [1,4,20–24], and previous studies specifically examining the fate of HiB5 cells following transplantation have demonstrated the cells’ ability to become both neurons and glia in an age- and region-specific manner [2,9–11]. Renfranz et al. [9] transplanted HiB5 cells into neonatal rat cerebellum and hippocampus, reporting the differentiation of as many as 20–25% of the transplanted cells. These results demonstrated the multipotential nature of the cells within their homotypic environment at young ages. Further experiments examined the fate of HiB5 cells transplanted into various regions of the adult rat brain. Lundberg et al. [2] transplanted HiB5 cells into the intact adult rat striatum and reported neuronal differentiation of 1–3% of transplanted cells, averaged over the entire striatum. Our data confirm that a small but significant percentage of HiB5 cells transplanted into the adult mouse striatum differentiate into neurons, and furthermore show that this percentage systematically
varies within the striatum. The calculation of the absolute percentage of donor cells that differentiate might be biased in a number of ways. However, none of the following considerations bias the finding of relative regional differences in differentiation. First, preferential survival of differentiated cells would lead to an overestimate of the percentage of transplanted cells that differentiate. Second, because \(^{3}H\)thymidine labeling becomes diluted with cell division, and if differentiated cells undergo more cell divisions than do undifferentiated cells (as has been proposed by Lundberg et al. [2]), the percentage of differentiated cells would be underestimated in our experiments because of their more dilute labeling. Finally, because we only counted heavily labeled cells that were three standard deviations above background labeling in order to avoid false positive identification, we probably underestimated the total number of HiB5 cells that survived and that differentiated. While relevant to the calculation of the absolute percentage of differentiation, none of these issues affects the relative degree of differentiation compared among different regions of the striatum. We therefore report our results as the normalized Differentiation Fraction, which represents the fraction of differentiated HiB5 cells in a given region relative to the fraction differentiated in the mid-lateral striatum. The studies presented here show that the relative fraction of HiB5 cells that differentiate into neurons and glia increases with proximity to the lateral ventricle, independent of the calculation of the absolute percentage of differentiation.

**Conclusion**

Our finding that both neuronal and glial differentiation increase exponentially with proximity to the subventricular zone raises the possibility of differentiation signals concentrated within the periventricular zone, but present throughout the striatum in a gradient. Further elucidation of factors that underlie the specific location-dependence of neuronal and glial differentiation by multipotent precursors in the adult brain may be important for the goal of repopulation and reconstruction of complex CNS circuitry.

**References**


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