Differentiation of adult hippocampus-derived progenitors into olfactory neurons *in vivo*

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NEUROGENESIS continues throughout adulthood in discrete regions. Proliferative zones include the subependymal zone[1-4], from where progenitors migrate along the rostral migratory pathway to differentiate into neurons in the olfactory bulb[4], and the hippocampal subgranular zone, where they migrate and differentiate into granule neurons[5-7]. Progenitors isolated from adult subependymal zone exhibit *in vitro* neurogenesis when stimulated with epidermal[8,9] or fibroblast growth factor[10]. Cultured adult rat hippocampal progenitors (AHPs) grafted to adult rat hippocampus show site-specific neuronal differentiation[11]. Here we investigate determinants of multipotentiality in the adult central nervous system, by grafting AHPs into homotypic (hippocampus) or heterotypic (the rostral migratory pathway) neurogenic sites or a heterotypic, non-neurogenic site (the cerebellum). We found that grafts into neurogenic, but not non-neurogenic sites, showed neuronal differentiation. Furthermore, AHPs grafted in the rostral migratory pathway migrated into the olfactory bulb, differentiating into tyrosine-hydroxylase-positive neurons, a non-hippocampus phenotype. These results reveal that AHP populations can respond to persistent neuronal differentiation cues in the adult central nervous system.

AHP cells in culture remain uncommitted, with only a small fraction differentiating into cells with mature neuronal or glial phenotypes[11,12]. To test whether AHPs cultured for over two years differentiate *in vivo* in response to endogenous local cues, cells labelled with 5-bromo-2′-deoxyuridine (BrdU), or an adenoviral vector carrying the *lacZ* gene, were grafted to adult rat hippocampus, cerebellum and rostral migratory pathway (RMP) (Fig. 1*a*). Control experiments, in which BrdU- and adenoviral-labelled cells were sonicated or freeze–thawed before grafting, showed no transfer of markers to endogenous cells[11]. The graft position, distribution of AHPs and their fate were examined at one and eight weeks post-grafting using BrdU- and β-galactosidase immunostaining. Immunostaining revealed that AHPs had survived at all time points in each target zone with no tumour formation (Fig. 1*b–g*). Of 75,000 AHPs grafted per site, 27,136 ± 5,924 (mean ± s.e.m.) were present in the olfactory bulb, 35,863 ± 1,123 in the hippocampus, and 26,584 ± 2,323 in the cerebellum 8 weeks after grafting. There was no significant difference (ANOVA, $\alpha = 0.05$) in AHP survival, indicating that graft site does not influence survival.

Examination of cell distribution at one week revealed the dispersion of AHPs in the hippocampus and cerebellum (Fig. 2*a*). However, AHPs in RMP migrated over 2 mm rostrally along the RMP and subependymal zone (SEZ) (Fig. 2*a*), where most (73%) cells were observed in SEZ, with some (19%) in the olfactory granule cell layer and few (8%) in the glomerular cell layer (Table 1*a*). Eight weeks post-grafting, cerebellar AHP distribution did not differ from that at one week (Fig. 2*b*), suggesting that dispersion rather than specific migration was occurring. In hippocampus, AHPs distributed in the CA1–3 regions and dentate gyrus[11]. In the olfactory bulb, AHPs migrated

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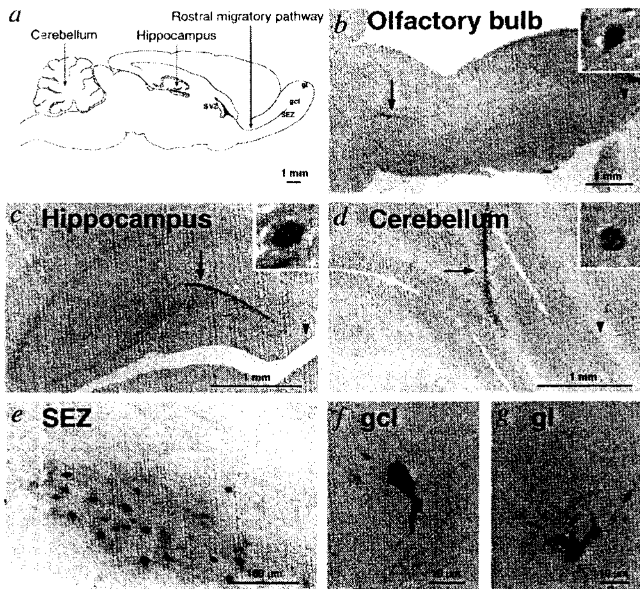

FIG. 1 Migration of AHPs grafted into the adult rat brain. *a*, Parasaggital view of the adult rat brain showing the three graft sites (arrows) with endogenous proliferative regions, the dentate gyrus and SVZ, shaded. BrdU-immunoreactive AHPs at eight weeks post-grafting (arrows) in *b*, the OB/RMP (a heterotypic neurogenic site); *c*, HC (a homotypic neurogenic site); and *d*, CB (a heterotypic non-neurogenic site). Insets in *b*–*d* show the most distant AHPs (arrowheads) from the graft site at higher power. AHPs immunoreactive for β-gal grafted to the RMP distributed along the SEZ (*e*) and in olfactory granule (gcl) (*f*) and glomerular (gl) (*g*) cell layers.

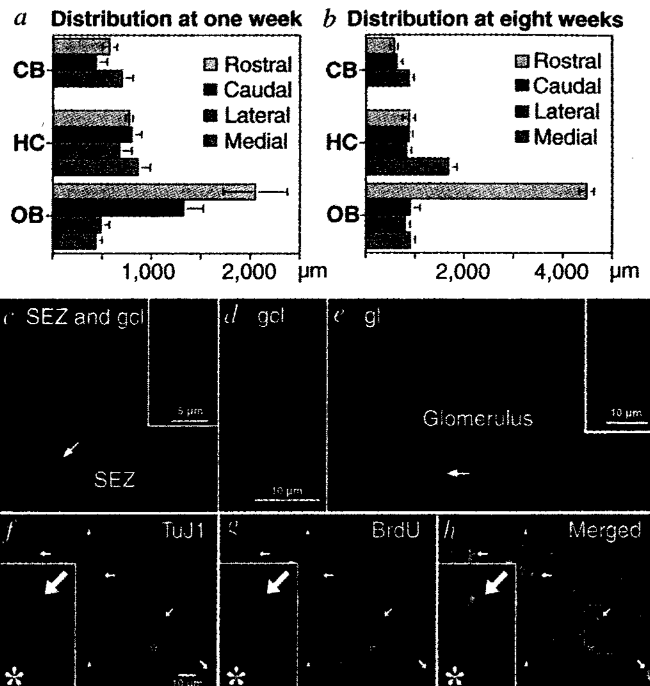

FIG. 2 Maximal distribution of AHPs grafted to CB, HC and OB from the graft centre in four anatomical axes at one (*a*) and eight (*b*) weeks post-grafting (±s.e.m.). One week post-grafting, some migrating BrdU-immunoreactive cells expressed association with PSA-N-CAM in the SEZ and gcl (*c*). At eight weeks post-grafting, association of grafted BrdU-immunoreactive cells with PSA-N-CAM was also found in gcl (*d*) and gl (*e*). Cells indicated by the arrows shown at higher power in the insets. Association of TuJ1 with BrdU-immunoreactive cells in RMP (*f*–*h*). Merged images of cells (*h*) immunostained for TuJ1 (*f*) and BrdU (*g*) showed the cells expressing both markers (arrows), BrdU only (arrowheads) or TuJ1 only (cell above the asterisk). Boxed areas are shown at higher-power magnification as insets at the bottom of *f*–*h*.

up to 5 mm rostrally (Fig. 2*b*), with only 9% remaining in SEZ, whereas most were found in the olfactory granule (69%) and glomerular (22%) cell layers (Table 1*a*). The extent and pattern of migration were similar to those for olfactory bulb development[4,13] and in adult neurogenesis[14–16], or following homotypic engraftment of freshly dissected cells from adult mice subventricular zone (SVZ; ref. 14). These results indicate that AHPs can respond to guidance cues present in the olfactory system, resulting in long-distance migration and laminar distribution similar to endogenous SVZ-derived progenitors of the host brain.

To investigate the long-distance migration of AHPs and the behaviour of the migrating cells, we examined the expression of polysialic acid neural-cell adhesion molecules (N-CAM; refs 15–18), glial fibrillary acidic protein (GFAP; an astroglia marker), and TuJ1 (a class III β-tubulin), previously used to mark neuroblasts[13]. Although expressed preferentially during embryonic development[19], polysialic acid N-CAM expression continues throughout adulthood in SVZ, olfactory system and hippocampus[15,16,20,21]. We detected polysialic acid N-CAM in the dentate gyrus, SVZ and olfactory system, but not in the cerebellum. Although AHPs were associated with polysialic acid N-CAM in hippocampus at one week, none was found at eight weeks post-grafting. Many AHPs were associated with polysialic acid N-CAM in chains along the SEZ at one week after grafting, as in endogenous SVZ cells[15,16] (Fig. 2*c*). By eight weeks, only a few N-CAM-associated AHPs remained within the SEZ, with most located in granule and glomerular cell layers (Fig. 2*d*, *e*), suggesting radial migration to superficial layers, where neuronal differentiation was observed (Fig. 3*b*–*d*). In addition to association with polysialic acid N-CAM, support for parallel behaviour of endogenous SVZ progenitors and grafted AHPs comes from the observation that at one week no AHP expressed GFAP in the RMP, although many BrdU-positive cells were TuJ1-positive (Fig. 2*f*–*h*), indicating that these migratory grafted AHPs in RMP were neuroblasts.

To determine whether AHPs can differentiate into neurons by eight weeks post-grafting, immunofluorescent labelling was performed with antibodies against tyrosine hydroxylase, calbindin, BrdU, NeuN, and GFAP. In the cerebellum, the heterotypic non-neurogenic site, AHPs did not express neuronal markers (Table 1*b*), suggesting that adult cerebellum may be deficient in neuronal differentiation cues, in contrast to that of the neonate[22,23]. Poor survival did not preclude differentiation, because AHP survival in cerebellum was equivalent in hippocampus and olfactory bulb. In hippocampus, the homotypic neurogenic site, only AHPs that migrated into neuronal layers of the dentate gyrus expressed the neuronal markers, NeuN (48%) (ref. 24) and calbindin (35%) (ref. 25; Table 1*b*). No tyrosine-hydroxylase-positive cells were detected in the hippocampus (Table 1*b*).

Although olfactory periglomerular neurons are reported to be immunopositive to calbindin and tyrosine hydroxylase[25,26] and immunonegative to NeuN[24], double-labelling revealed that calbindin and tyrosine hydroxylase are expressed in separate, non-overlapping periglomerular subpopulations (Fig. 3*b*, *c*), whereas olfactory granule cells are NeuN-positive but calbindin-negative. When grafted to RMP, the heterotypic neurogenic site, 10% of AHPs within the glomeruli expressed calbindin (Fig. 3*b* and Table 1*b*), and 16% of AHPs within the granule cell layer were NeuN-positive (Fig. 3*d* and Table 1*b*). Furthermore, 7% of AHPs in the glomeruli expressed tyrosine hydroxylase (Fig. 3*c* and Table 1*b*), a marker for dopaminergic neurons. Tyrosine-hydroxylase-positive neurons are not found in hippocampus or in AHPs in culture, or in AHPs grafted to the hippocampus (data not shown). Expression of tyrosine hydroxylase indicates that the AHP population may differentiate in a multipotential fashion after grafting in response to specific local cues. AHPs immunostaining for β-galactosidase displayed an interneuron-like morphology in granule and glomerular

TABLE 1 Distribution of grafted cells

(*a*) Regional distribution of AHPs in olfactory bulb

	SEZ	Granule cell layer	Glomeruli	Total
1 week	258* (73%)	67 (19%)	29 (8%)	354 (100%)
8 weeks	83 (9%)	611 (69%)	201 (22%)	895 (100%)

(*b*) Phenotypic distribution of AHPs in all grafted areas

	Olfactory bulb			Hippocampus			Cerebellum	
	Glomeruli	Granule cell layer	SEZ	Granule cell layer	Area CA1	Area CA3	Granule cell layer	Purkinje cell layer
BrdU⁺	203†	204	205	102	105	101	100	50
TH⁺	7%‡	0%	0%	0%	0%	0%	0%	0%
Calb⁺	10%	0%	2%	48%	3%	4%	0%	0%
BrdU⁺	201†	202	204	100	103	102	102	60
NeuN⁺	0%‡	16%	0%	35%	0%	0%	0%	0%
GFAP⁺	21%	25%	29%	4%	34%	31%	28%	17%

a, Grafted cells at one and eight weeks after implantation into the rostral tip of RMP were counted from BrdU-immunoreactive sections in the same olfactory bulb regions used for data collection in *b* (one section per animal; five animals per time point). *b*, Eight weeks post-grafting, sections including olfactory bulb, hippocampus and cerebellum were triple-labelled with neuronal markers (tyrosine hydroxylase, TH; calbindin, Calb; and NeuN) and an astrocytic marker (GFAP) and analysed by confocal microscopy. BrdU-immunoreactive cells were counted in regions outside the injection site in each target zone. Because of the high abundance of BrdU-positive cells within neuronal layers, an upper limit of 200 sampled cells was imposed. SEZ, subependymal zone.

* The total number of BrdU⁺ cells counted per area.

† The total number of BrdU⁺ cells counted per area.

‡ Percentage of cells double-labelled for BrdU and the indicated marker.

cell layers (Fig. 1*f*, *g*). The observations that at one week grafted AHPs in RMP are GFAP-negative but TuJ1-positive, and associated with polysialic acid N-CAM, and that at eight weeks the grafted AHPs in the olfactory bulb express tyrosine hydroxylase, calbindin and NeuN, indicate that AHPs can behave as endogenous progenitors.

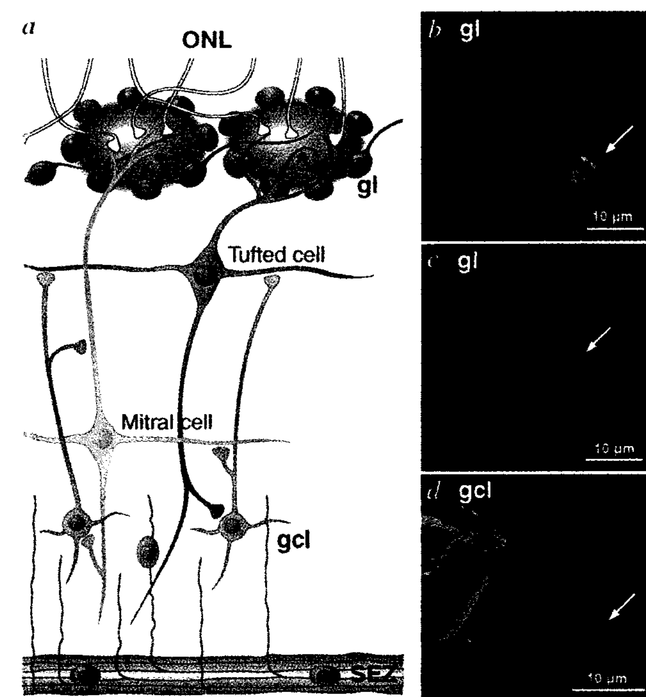

FIG. 3 Differentiation of grafted AHPs in OB. Schematic of adult OB (modified from ref. 30). ONL, olfactory nerve layer; gl, glomerular cell layer; gcl, granule cell layer; SEZ, subependymal zone (*a*). Neuronal phenotype of AHPs at eight weeks (*b*–*d*). Calbindin- (red) and TH- (blue) immunoreactive periglomerular neurons in the gl of OB (*b*, *c*). BrdU-immunoreactive AHPs express calbindin (yellow; arrow in *b*) and TH (light blue; arrow in *c*), a non-HC phenotype. NeuN-immunoreactive neurons (purple) forming the gcl of OB (*d*). Yellow indicates a grafted cell (arrow) in the gcl, double-labelled for NeuN and BrdU; turquoise indicates GFAP immunoreactivity.

Previous studies have used fetal or neonatal tissue to investigate the regional environmental cues underlying neuronal differentiation[23,27,28], but our results indicate that the adult nervous system contains progenitor cells that can be expanded *in vitro* and can respond to persistent cues in adult brain for survival, migration and neuronal differentiation. Furthermore, the existence of AHPs positive for tyrosine hydroxylase in the olfactory bulb suggests that regional cues in the adult central nervous system (CNS) can direct differentiating neurons down specific phenotypic pathways. Our observations also suggest that regional cues within the adult CNS may direct phenotypic differentiation. Identifying the conditions for phenotypic differentiation of adult CNS progenitors may provide new strategies for treatment of neurodegenerative diseases and trauma. □

Methods

Grafting of AHPs in the brain. AHPs (75,000 cells in 1.5 μl) labelled with BrdU and/or infected with an adenovirus vector expressing cytoplasmic *E. coli lacZ* (ref. 11) were stereotaxically injected into the rostral tip of RMP (AP +4.8, ML ±2.0, DV −3.0 from dura), cerebellum (CB) (AP −10.4, ML ±2.0, DV −4.0 from dura) and hippocampus (HC) (AP −3.5, ML ±3.0, DV −3.9 from skull, with nose bar at 5 mm up) of adult female Fischer-344 rats. (AP, Anteroposterior axis; ML, mediolateral axis; DV, dorsoventral axis.)

Immunostaining and analysis of brain tissues. At one (short-term; $n = 10$) and eight (long-term; $n = 10$) weeks post-grafting, animals were perfused (4% paraformaldehyde), the brains sectioned (40 μm), and immunoperoxidase-stained for BrdU and β-galactosidase (β-gal)[11]. For immunofluorescent staining, sections were pretreated for BrdU detection and stained[11] with mouse anti-NeuN (1:10; from R. Mullen[24]), rabbit antibody against calbindin-D (28K) (1:1,000; Swant), rabbit anti-GFAP (1:250; Chemicon), mouse antibody against tyrosine hydroxylase (TH) (1:250; Boehringer Mannheim), mouse anti-MenB (to detect polysialic acid (PSA)-N-CAM residues; 1:1,000 with biotin–streptavidin amplification; from G. Rougon[29]), mouse anti-TuJ1 (1:1,000; from A. Frankfurter) and mouse anti-BrdU-FITC (1:20; Boehringer Mannheim). The secondary antibodies used in triple-labelling were donkey anti-species Texas red, Cy3 or Cy5 (1:250; Jackson Immunoresearch). Sections were imaged using a Bio-Rad MRC1000 confocal microscope.

Quantification. To examine the distribution of grafted cells, semiserial sections containing the OB, HC or CB (a 1 in 6 horizontal or coronal series) were stained immunohistochemically for BrdU and the number of BrdU-immunoreactive cells was quantified using stereological procedures[11]. Quantitative data were compiled and analysed statistically. Error was expressed as the standard error of the mean (s.e.m.). The vector distance (straight-line distance) between the graft centre and the most distant AHP was measured (in micrometres) in the four anatomical axes from horizontal OB sections (five grafts, three sections per graft, one measurement per axis per graft), coronal hippocampal sections (five grafts,

three sections per graft, one medial/lateral measurement per graft) and cerebellar sections (five grafts, one section per graft, six medial/lateral measurements per graft). For coronal sections, rostrocaudal migration was calculated from the distance between sections.

Received 1 April; accepted 2 September 1996.

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ACKNOWLEDGEMENTS. We thank P. Eriksson, L. J. Fisher, M. Gage, M. B. Luskin, T. D. Palmer and H. K. Raymon for helpful criticism of the manuscript and S. Forbes for his help with animal care. This work was supported by separate grants from the NIA, NINDS, American Paralysis Association, Hollfelder Foundation, International Spinal Research Trust and the MRC of the Academy of Finland.

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